

Exploring and mapping genetic variation in
wild barley, *Hordeum spontaneum*

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Abstract

Wild barley represents an important genetic resource for cultivated barley, which has a narrowed gene pool due to intensive breeding. Therefore, it is imperative to study the genetics of different traits in wild barley, if it is to be used for cultivar improvement. This thesis describes studies of different aspects of diversity, as well as linkage, QTL and association mapping in wild barley. The natural populations that were studied were collected in environmentally diverse Israel. Using a cross between genotypes from contrasting habitats, a linkage map was produced. Due to unexpectedly high heterozygosity of one of the parents the map covered approximately 45% of the barley genome. This map was subsequently utilised in all other studies in this thesis. Genetic diversity was estimated within and between 21 populations. This was correlated with phenotypic and environmental diversities of the same populations. Genetic diversity was loosely connected with environmental diversity. In general, phenotypic diversity did not correlate with either one. Some markers were ecotype-specific; some of these were mapped and correlated significantly not only with environmental but also with phenotypic traits. Using the same populations, correlations between mapped markers and phenotypic traits were calculated. The significant associations were then compared with QTL found in an earlier study. Many of these associations mapped to the same regions as the QTL and therefore these two approaches of mapping traits complemented each other. Lastly, the map was used to locate QTL for dormancy in wild barley. Five QTL were found of which three map to regions where dormancy QTL has been found earlier in cultivated barley. For further research, another cross should be made to produce a map with larger coverage of the genome. The results of this thesis add to the characterisation of the diverse wild barley populations from Israel, which is important to the future improvement of cultivated barley.

Chapter 1

Many years have passed since those summer days
Among the fields of barley
See the children run as the sun goes down
Among the fields of gold
You'll remember me when the west wind moves
Upon the fields of barley
You can tell the sun in his jealous sky
When we walked in the fields of gold

Sting – Fields of gold



General Introduction

Hordeum spontaneum, wild barley

Hordeum spontaneum (wild barley) is the ancestor of cultivated barley. It belongs to the *Poaceae*-family of grasses and within it to the *Triticeae*-tribe. *Triticeae* is a temperate plant group mainly concentrated around Central and Southeastern Asia, although the species belonging to it are distributed around the world. *Triticeae* includes many economically important cultivated cereals and forages but also about 350 wild species. The wild species are of great interest as potential gene donors for commercial breeding.

The genus *Hordeum* includes 30 species (Von Bothmer 1992), both annuals and perennials. Wild barley can be winter or summer annual depending on the local climate. It is diploid ($2n=14$) and mainly inbreeding, although some populations have a higher rate of outcrossing. On average this is 1.6%, varying from 0 to 9.6%, depending on the environment; it is significantly higher in the mesic than in the xeric regions (Brown *et al.* 1978a).

The wild annual *Hordeum* species grow in open habitats with comparatively low competition from other species (Von Bothmer 1992). Wild barley is distributed over the eastern Mediterranean area and

in Southwest Asia across a wide range of climates and soils. It is particularly common in the Near East Fertile Crescent (Zohary 1969). In general, wild barley is not tolerant to extreme low temperatures and is rarely found above 1500 m altitude. However, it is more drought resistant than the wild wheat and penetrates relatively deeply into the warm steppes and deserts (Zohary & Hopf 1988).

Wild barley and cultivated 2-rowed barley have quite similar morphology. The most notable differences are wild barley's brittle rachis and its hulled grain. Six-rowed barley has evolved during domestication, the trait being controlled by a single gene on chromosome 2 (Komatsuda *et al.* 1999; Tanno *et al.* 2002). Wild barley is the only wild *Hordeum* species that can produce fully fertile hybrids (with normal chromosome pairing and segregation in meiosis) when crossed with cultivated barley. Hybrids can also be formed in nature when these two occur at the same location (Asfaw & Von Bothmer 1990).

The collection of wild barley from Israel was obtained between 1974 and 1976 (Nevo *et al.* 1979). During that period over a thousand different accessions were collected from the whole of Israel. Sampling was done from various habitats like road and mountainsides,

fields and deserts. Latitude, longitude, altitude and different rainfall and temperature variables were reported from the nearby weather stations. This collection has been maintained in the Institute of Evolution, Haifa, Israel by prof. E. Nevo and his co-workers. During successive years, more samples were added to the collection; populations from Tabigha (Nevo *et al.* 1986a) and Evolution Canyon (Nevo 1995). Accessions from this collection in Haifa have been distributed around the world. However, because of the high number of accessions available, there is hardly any overlap on the material used in the different studies. Because these samples have been collected from widely diverse habitats, the collection represents a rich source of phenotypic and genetic variation, which can be used to study ecophysiological issues both at the phenotypic and genotypic level. In addition, the wild barley accessions of this collection are a potential source of variation to be tapped by plant breeders for the improvement of cultivated barley.

Variation in wild barley from Israel

The genetic variability of wild barley has been described in many studies. So far, the only research including all the collected accessions from Israel has been conducted by using isozymes (Brown *et al.* 1978b; Nevo *et al.* 1979). After the invention of polymerase chain reaction

(PCR) to amplify DNA, many different types of molecular markers have been developed. Wild barley from Israel has also been studied utilising several of these PCR-based methods: RAPDs (Baum *et al.* 1997; Owuor *et al.* 1997, 1999), AFLPs (Pakniyat *et al.* 1997, Turpeinen *et al.* 2003) and SSRs (Turpeinen *et al.* 2001). RFLPs (Chalmers *et al.* 1992), retrotransposon copy numbers (Kalendar *et al.* 2000) and genome size (Turpeinen *et al.* 1999) were also used in studying wild barley diversity within Israel.

Studies with wild and cultivated barley have shown that there is more variation within the wild than in the cultivated barley (*e.g.* Saghai Maroof *et al.* 1995), although in some cases the opposite has been reported for some isozymes and mitochondrial DNA (Nevo, 1992). The larger genetic variation within wild barley gives the opportunity to use this variation for breeding purposes.

The genetic diversity of wild barley is correlated with its geographic distribution on a large scale, between countries. But it is not evident on the micro-geographic scale within Israel. For example, Song & Henry (1995) found that some of the accessions that were identical genetically were geographically widely scattered and of contrasting habitat.

Resistance to biotic and abiotic stresses has been the main area of study on the phenotypic variation in wild barley, as these are important targets for improvement in barley breeding (Ellis *et al.* 2000). Wild barley accessions from Israel have been screened for resistance

to several plant pathogens (*e.g.* Moseman *et al.* 1983, 1990; Ordon *et al.* 1997). Several lines were resistant to *Erysiphe graminis* and *Puccinia hordei* as well as soil-borne mosaic-inducing viruses (Moseman *et al.* 1983, 1990; Ordon *et al.* 1997). Other agronomically interesting traits include a range of physiological and chemical traits (*e.g.* Ellis *et al.* 1993; Forster *et al.* 1994; Nevo *et al.* 1993a, 1992, 1985), abiotic stress tolerance (*e.g.* Nevo *et al.* 1993b, Pakniyat *et al.* 1997, Robinson *et al.* 2000) and morphological characters (*e.g.* Gutterman *et al.* 1996, Nevo *et al.* 1984, Giles 1990).

Quantitative trait loci (QTL) mapping and wild barley

Linking genetic information with phenotypic measurements is vital if wild barley is to be used for barley improvement. This can be done either by mapping quantitative trait loci (QTL mapping), association mapping or both. Both of these methods are based on associations between quantitative traits and marker alleles, which is due to linkage disequilibrium between the alleles at marker loci and trait controlling loci; both have their advantages and disadvantages.

QTL mapping requires the construction of a linkage map using a cross between phenotypically divergent accessions. In the offspring of such a cross, association between a trait and

marker alleles arises from linkage between marker loci and loci controlling the trait. By identifying these associations, the method allows the location of genomic regions on a marker linkage map that most likely contain genes involved in the trait (the QTL). The results of QTL mapping provide the most likely position of the QTL, together with an estimate of the allele substitution effects (the additive effect) and so called 'supportive intervals' that roughly correspond to confidence intervals for the QTL map positions. The precision that can be attained in QTL mapping, both with respect to positioning on the map and the estimated substitution effect, depends largely on two factors, *i.e.* the size of the mapping population and the genetic variation that is attributable to a particular QTL. In general, using a mapping population of size below 400, confidence intervals for QTL positions cover map distances as large as 20 to 60 centimorgans. More precise location (fine mapping) of QTL requires different approaches, such as the construction of backcross inbred lines, containing small introgressed 'donor' genome fragments in a homogeneous recipient genetic background. From a breeding perspective a potential disadvantage of QTL detection in mapping populations is that the results obtained from a particular cross cannot *a priori* be extrapolated to other crosses because the magnitude of QTL effects and the detectability of QTL depends on the genetic background. Nonetheless, several studies have shown that certain QTL

regions may be common to several crosses. Interspecific crosses between wild and cultivated barley have been made (Graner *et al.* 1991, Pillen *et al.* 2000, Ramsay *et al.* 2000) and some QTL studies have been conducted using such an interspecific cross (Ellis *et al.* 1997, Huang *et al.* 2001, Backes *et al.* 2003, Baum *et al.* 2003).

Another way of screening and locating useful traits in wild barley populations is to look for associations between the traits and previously mapped markers among the wild populations (*e.g.* Nevo *et al.* 1984, Pakniyat *et al.* 1997). The advantages are notable as the development of mapping populations is not required and the study is not limited to a single cross between two individual parent lines that might not express sufficient variation in the traits of interest. In contrast to QTL mapping, in which the difference between the two parental genotypes is analysed, association mapping (also referred to as linkage disequilibrium (LD) mapping) relies on the association between traits and marker alleles across a large set of populations or accessions. Associations between the alleles of markers and QTL in a germ plasm collection may arise in various ways. First, identity by descent of the alleles of tightly linked loci may be the cause. It is this type of association, or linkage disequilibrium, that association mapping tries to uncover. In a classical mapping population the degree of linkage disequilibrium between linked loci depends on the recombination events

that have taken place during a single or a few meioses following the cross between the parents, whereas in a germ plasm collection linkage disequilibrium reflects the recombination events that have accumulated during the history of the germ plasm. Usually this history traces back to an unknown point in time. For these reasons the amount of LD that can be encountered depends on the history of the germ plasm, its population structure and its mating system (outbreeding *versus* inbreeding). In addition to linkage, associations in a germ plasm collection may also arise by chance (*i.e.* admixture, outcrossing events etc.), resulting in LD between the alleles of unlinked or loosely linked loci. Therefore, observed associations between traits and marker alleles in a population survey should be interpreted with care. It is helpful when the markers used in an association study have been mapped previously, so that the map distance over which significant linkage disequilibrium between markers extends, can be estimated. Combining and comparing the results of association studies with those obtained from QTL mapping may then lead to a better and more reliable identification of the genomic regions that contain the trait-controlling genes. From a plant breeding perspective, an obvious advantage of association mapping over QTL mapping exercises is that the observed associations are not confined to a particular cross, but hold over a wild range of a crop's germ plasm. An additional advantage of association mapping is the potential high precision of

QTL location, as compared to QTL mapping. This is because in a germ plasm survey, significant associations between the alleles of linked loci usually do not extend beyond a few centimorgans (Kraakman *et al.*, 2004); in outbreeders the distance over which linkage disequilibrium extends may be even much smaller (see Rafalski & Morgante 2004 for a recent overview). So, if a trait is associated with a few closely linked markers that cover a tiny chromosomal segment, but markers outside that segment do not show association with the trait, one can infer that the hypothesised QTL is within that small segment.

Outline of this thesis

As well as exploring the genetics of wild barley from Israel, this thesis forms the genetic backbone of a bigger research program, which was set up to study relative growth rate and its components in wild barley from Israel. The other parts of the research program included physiological research done at the Utrecht University (Van Rijn, 2001), plasticity under different nutrient conditions (Elberse, 2002) and selection and adaptation (Verhoeven, 2003). Both latter studies were conducted at the Netherlands Institute of Ecology, Heteren, with some of the field work taking place in Israel. The resulting marker data and linkage map from this thesis were shared with all of these subprojects and QTL studies for a broad array of traits were

conducted to unravel the genetic basis of relative growth rate (Elberse, 2002; Poorter *et al.* 2004; Verhoeven *et al.* 2004a). The overall aim of this program was to unravel the genetic basis of the slow *versus* fast growing 'syndrome' in wild barley and to investigate how the various physiological components of relative growth rate respond to a change in environmental conditions, both natural and controlled. A hypothesis to be tested was the existence of a 'major switch', possibly controlled by a single gene, which determines whether a genotype is slow or fast growing; such a switch might control several components underlying relative growth rate. Briefly, the results, obtained by QTL analyses in various environments, showed that growth rate is influenced by several genes, some of which have an effect on more than one component of growth rate and that the size of the QTL effects may be environmentally dependent thus leading to QTL by environment interaction. The overall picture, however, indicated that the direction of the QTL effects does not change between environments that are characterised by different levels of nutrient availability.

The objectives of the present thesis were

1. to assess the genetic diversity between and within wild barley populations,
2. to study, at the population level, the associations between physiological traits and DNA markers and to see to what extent these are in

accordance with the results of earlier QTL studies, and

3. to study the genetics of dormancy in wild barley by means of a QTL mapping approach.

Because several of these issues, as well as the QTL studies performed in the other projects of the program, require a linkage map, a molecular marker linkage map, based on an intraspecific wild barley cross, has been constructed. Such a linkage map is also vital for future identification of agronomically relevant genes that are present in this germ plasm, especially because wild barley is far more variable than cultivated barley.

As there was no previous detailed genetic background information of the particular accessions that were used in this and the other theses, it was of major importance to estimate the variation within and between all the studied populations. This undertaking has greatly enlarged the understanding of these populations and given new views for further research and has provided the basis for the choice of the parents to be crossed for linkage and QTL mapping.

From a breeding perspective it is equally important to screen all the populations for agronomically important traits and identify the genomic regions that harbour the underlying genes. Studies that uncover associations between traits and DNA markers at the level of germ plasm collection provide a gateway toward efficient tapping of this genetic resource for breeding purposes. This type of screening is much faster than

producing numerous crosses and recombinant inbred lines for QTL mapping. The most promising and interesting accessions can thereafter be crossed so that high quality QTL mapping populations can be produced for fine mapping of QTL, and possibly, cloning of the underlying genes.

Seed dormancy is extremely important for the survival of wild species as germinating during 'wrong' season can be fatal. It is just as important in cultivated barley, as too dormant cultivars will require after-ripening storage before germination can occur, and completely non-dormant cultivars might germinate before harvesting, thus significantly decreasing the quality and value of the harvested crop. So far, several QTL for dormancy have been identified in cultivated barley and their variation and expression has been studied in minute detail (*e.g.* Romagosa *et al.* 1999, Gao *et al.* 2003). Additional dormancy QTL from wild barley will aid the designing of an optimally dormant cultivar in providing more genetic variation to work with.

Chapter 2 presents the first wild barley genetic linkage map. The linkage map was constructed using an F₂ population from a single pair cross between two accessions from divergent populations.

In Chapter 3, the genetic variation in 21 wild barley populations from Israel is estimated using AFLP markers. Phenotypic and environmental data were also used to establish the general pattern of variation within and among these

populations. Correlations between these three different levels of variation were studied as well as associations between individual mapped markers and phenotypic and environmental variables.

In Chapter 4, map-based AFLP markers were associated with the variation in growth traits in the 21 wild barley populations and compared with the QTL reported by Poorter *et al.* (2004).

In Chapter 5, the genetic basis of seed dormancy in wild barley is studied. The experimental work was performed using F_4 lines derived from F_2 plants of the above-mentioned cross (Chapter 2).

Chapter 6 gives a summarising discussion on the most important conclusions from the previous chapters.

Chapter 2

Do you remember the Shire, Mr. Frodo?
It'll be spring soon, and the orchards will be in blossom,
and the birds will be nesting in the hazel thicket.
And the whistle in the summer barley in the lower fields.
And eating the first of the strawberries with cream.
Do you remember the taste of strawberries?

J.R.R. Tolkien – The Return of the King



A GENETIC LINKAGE MAP OF WILD BARLEY (*Hordeum spontaneum*)

Tytti K. Vanhala & Piet Stam

Abstract

*A molecular marker map of wild barley (*Hordeum spontaneum*) was constructed using an F_2 population derived from the cross 'Ashkelon' x 'Mehola'. Thirteen SSR markers and 22 AFLP primer combinations were used, yielding 384 polymorphic markers of which 202 were eventually mapped to 11 linkage groups. The map coverage was 445 cM, corresponding to an average distance of 2.2 cM between adjacent markers. To assign the linkage groups to known barley chromosomes, 33 AFLP markers, which were previously mapped in four different cultivated barley crosses and the SSRs were used as chromosome-specific markers. Due to heterozygosity of one of the parental plants used in this cross the map is estimated to cover approximately 45% of the wild barley genome. This map is the first intraspecific molecular linkage map of wild barley.*

Introduction

Wild relatives of crop plants are important resources for crop improvement (e.g. Tanksley & Mc Couch 1997). Intraspecific crosses are important for detection and mapping of quantitative trait loci (QTL) controlling agronomic traits within wild species. Crosses between wild and cultivated species are important for transferring desired QTL alleles to cultivars and/or advanced breeding lines.

Wild barley (*Hordeum spontaneum*) is the ancestor of cultivated

barley (Zohary 1969). Interspecific hybrids between wild and cultivated barley are fully fertile. This and the diversity of wild barley make it attractive for the genetic improvement of barley cultivars. Wild barley is diploid ($2n = 14$) and although some outcrossing has been detected in the field, it is a predominantly self-fertilising plant species (Brown *et al.* 1978a).

Linkage maps are important for various purposes such as the detection and localisation of quantitative trait loci. Linkage maps based on several crosses of cultivated barley have been published

over the years (e.g. Becker *et al.* 1995, Qi *et al.* 1998, Yin *et al.* 1999). Also crosses between cultivated and wild barley have been used to construct linkage maps (Graner *et al.* 1991, Laurie *et al.* 1993, Pillen *et al.* 2000, Ramsay *et al.* 2000). Even though wild barley has been extensively studied, up to now no linkage map has been constructed based on an intraspecific cross.

The AFLP method (Vos *et al.* 1995) has become a popular tool for linkage mapping. The markers obtained using this method are easily obtainable in large numbers, thus giving a genome wide coverage of markers within a relatively short time period. Their disadvantage is that they are mainly dominant. This may cause problems in assembling a linkage map if the population used is an F_2 . The problem with mapping dominant markers in an F_2 arises with markers that are in repulsion phase. First, the recombination frequency of repulsion phase markers is estimated with less precision than from coupling phase markers. Secondly, the maximum likelihood (ML) estimator for repulsion phase markers is substantially biased downwards whereas the ML estimator for coupling phase markers is virtually unbiased. A detailed account of the problems with mapping dominant markers in an F_2 is given by Knapp *et al.* (1995) and Maliepaard *et al.* (1997). These problems usually result in two separate maps, a paternal and a maternal one, each containing markers in coupling phase only.

Co-dominant markers such as microsatellites (SSRs), as well as co-dominantly scored AFLP markers can be used as anchors to integrate maternal and paternal linkage maps.

In this study we have constructed the first linkage map from an intraspecific wild barley cross using AFLP and SSR markers.

Material and methods

Plant material

A cross was made between a plant from accession 28-77 ('Ashkelon') and a plant from accession 22-28 ('Mehola') of wild barley. The accessions were selected on basis of their growth characteristics as well as their habitat locations in Israel (Van Rijn *et al.* 2000); Ashkelon is situated in the Mediterranean coast whereas Mehola is located in the Jordan Valley (Brown *et al.* 1978b; Figure 1 in Chapter 3). Because of differences in their environmental conditions, the two accessions were expected to differ considerably both phenotypically and genotypically.

The linkage map from this cross was constructed to enable the mapping of QTL underlying a suite of growth-related traits including RGR (initial relative growth rate), SLA (specific leaf area), LAR (leaf area ratio) and photosynthesis rate.

Eight F_1 plants were self-fertilised to obtain eight F_2 sub-families.

To prevent outcrossing, which in 'Ashkelon' was observed to range from 0.1% to 10.0% and in 'Mehola' from 0% to 7.4% (Brown *et al.* 1978a), all spikes were bagged in paper bags at anthesis. A total of 233 F₂ plants were grown and used as a mapping population. Because the structure of this mapping population was kept track of, F₁ plants that were homozygous for any marker could easily be traced and the F₂ data could be corrected as appropriate (see 'Data Analysis').

DNA was isolated from two week old leaves using the CTAB method (Ausubel *et al.* 1999).

AFLP and SSR markers

Seventeen primer combinations were selected: E33M54, E33M61, E35M48, E35M54, E35M61, E38M55, E38M58, E42M51 and E45M55 (Qi & Lindhout 1997), E37M32, E37M33, E40M38, E41M32, E41M40, E42M32, E42M40 (Becker *et al.* 1995) and E31M55 (Table 1). The AFLP protocol was essentially as described in Vos *et al.* (1995), with the exception of diluting the secondary template only 10 times. The DNA was double digested with the restriction enzymes EcoRI and MseI. The EcoRI specific primers were labeled with either 700 or 800 nm infrared dye (IRD700, IRD800) for detection with a Licor automated laser sequencer (Li-Cor Inc., Lincoln, NE, U.S.A.). In addition, markers generated by the primer combinations E32M61, E33M55, E39M61, E42M48 and

E38M54 (Qi & Lindhout 1997) were genotyped co-dominantly by Keygene B.V., Wageningen, The Netherlands.

A set of 13 SSR markers were used (Ramsay *et al.* 2000; Liu *et al.* 1996). The primers were labeled with either IRD700 or IRD800 for Licor sequencer (Li-Cor Inc., Lincoln, NE, U.S.A.). Approximately 20 ng of template DNA was used in PCR reactions which consisted further of 1 x PCR buffer, 0.5 U of AmpliTaq polymerase (Perkin Elmer Inc., Wellesley, MA, U.S.A.), 200µM dNTPs and 1 pmol of forward and reverse primer. The reaction volume was 10µl. Four different PCR programs were used for amplification as described by Ramsay *et al.* (2000).

Data analysis

The AFLP markers from 17 primer combinations were scored dominantly as the absence or presence of an amplification product. The scoring was done by eye with the help of the program Cross Checker (Buntjer 1999). The markers from five primer combinations generated and scored by Keygene B.V. were scored co-dominantly using the QuantarTMPro software (http://www.keygene-products.com/html/index_products.htm), which enables a distinction between heterozygous and homozygous state based on band intensity. The AFLP marker names were designated from the primer combination and size of the

Table 1. List of primers and adapters used^a.

Primers/adapters		Sequences
<i>MseI</i> adapter		5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
M00 (universal primer)		5'-GATGAGTCCATGAGTAA-3'
<i>MseI</i> +1 primer	M02	M00 + C
<i>MseI</i> +3 primers	M32	M00 + AAA
	M33	M00 + AAG
	M38	M00 + ACT
	M40	M00 + AGC
	M48	M00 + CAC
	M51	M00 + CCA
	M54	M00 + CCT
	M55	M00 + CGA
	M58	M00 + CGT
	M61	M00 + CTG
<i>EcoRI</i> adapter		5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
E00 (universal primer)		5'-GACTGCGTACCAATTC-3'
<i>EcoRI</i> +1 primer	E01	E00 + A
<i>EcoRI</i> +3 primers	E31	E00 + AAA
	E33	E00 + AAG
	E35	E00 + ACA
	E37	E00 + ACG
	E38	E00 + ACT
	E40	E00 + AGC
	E41	E00 + AGG
	E42	E00 + AGT
	E45	E00 + ATG

^a Full listing of primer nomenclature and sequences are to be found from:

<http://www.keygene.nl/html/nomenclature.htm>

amplification product. SSRs were scored co-dominantly.

Inspection of the overall segregation data revealed that a large proportion (36%) of the markers showed significant distortion from their expected segregation ratios. Many of these skewed markers yielded suspect estimates of recombination frequency (ranging from 0.70 to 0.95) with non-distorted markers. A closer inspection of the data revealed that some of the eight F₂ sub-families did not segregate for the majority of these severely distorted markers. A non-segregating F₂ subfamily indicates that the

parental F₁ plant was homozygous for that particular marker. From this observation, *i.e.* some F₁ plants being homozygous and some being heterozygous for a particular marker, we concluded that one of the parents of our cross must have been heterozygous at such a marker locus. The consequence of neglecting this heterogeneity of F₁ plants would be twofold, *i.e.* (1) a severe distortion of segregation, and (2) a serious over-estimation of the recombination frequency between distorted and non-distorted markers (Stam, unpublished results). This is exactly what we observed in the overall,

complete, data set.

Having observed the above feature of the data and being aware of its consequences, we corrected the raw data as follows. Each marker was checked for its segregation in each of the eight F_2 sub-families. If a marker was non-segregating in a particular sub-family, all the data for that marker in that sub-family were removed (set to "missing"). As a result of this correction, for many markers the majority of the data points were lost, leaving insufficient linkage information to reliably map them. Another consequence of the correction is that some data were removed not because of genuine non-segregation, but because of non-segregation that occurred just by chance. Sub-family size ranged from 14 to 50 (average 29.1) so it is expected that in some small sub-families the observed non-segregation has been the result of genetic sampling rather than homozygosity of the parental F_1 plant. Because the correction described above also influences any genuine skewness of segregation, such as might arise through some pre- or post-zygotic selection mechanism, the corrected data leave little room for sensible interpretation of the remaining distorted segregation ratios.

With the corrected data set a linkage map was constructed using the algorithm described by Stam (1993), as implemented in the JoinMap 3.0 software package (Van Ooijen and Voorrips 2000). Linkage groups were assigned using a LOD threshold of 5.0. Kosambi's mapping function was used to calculate map

distances. Before ordering the markers within each of the linkage groups, the data were split into three sub-sets, *i.e.* (1) the dominant 'Ashkelon'-specific markers, (2) the dominant 'Mehola'-specific markers and (3) the co-dominant markers. For each of these sub-sets an ordering was calculated within linkage groups, which could be used as a so-called 'fixed order' in the final merged data set. The reason to first establish the marker order within paternal and maternal maps as well as for the anchor co-dominant markers is that these sub-sets represent the most reliable part of the linkage information in the data. The marker order in the merged map should not be in conflict with the ordering in these constituent maps.

To assign the linkage groups to known barley chromosomes, SSR loci (Ramsay *et al.* 2000) as well as AFLP markers in common with earlier maps from several cultivated barley populations L94 x Vada' (Qi *et al.* 1998), 'L94 x 115-6' (Lindhout, personal communication), 'Apex x Prisma' (Yin *et al.* 1999) and 'Proctor x Nudinka' (Becker *et al.* 1995) were used.

Results and discussion

By using 22 primer combinations we obtained 348 polymorphic AFLP markers, of which 151 (43%) were scored co-dominantly. The 13 SSRs were all informative without further adjustment of the original PCR conditions. However, only

five from the 13 could be scored without problems. Two null alleles were encountered as well as four alleles for which amplification was repressed by one or two other alleles. Six SSRs were heterozygous in the Ashkelon and two in the Mehola parent.

The adjustment of the raw data (see 'Data Analysis') revealed that the 'Ashkelon' parent must have been highly heterozygous; 59% of the 'Ashkelon'-specific markers did not segregate in at least one F₂ sub-family. Heterozygosity was less prominent in the 'Mehola' parent; 7% of the 'Mehola'-specific markers did not segregate in at least one F₂ sub-family.

After adjustment, 202 markers (56%) could be mapped without problems. The remaining ones (159) could not be mapped, mainly because they would not link to any other marker, as a result of the large proportion of missing genotype scores. Therefore, due to the heterozygosity in at least one of the parents of this cross, about 45% of the markers contained no useful linkage information.

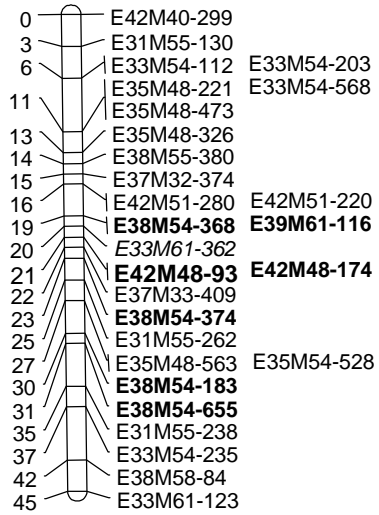
The linkage map is shown in Figure 1. It contains 202 markers

distributed over 11 linkage groups. Except for linkage group U1, each linkage group contains both dominant and co-dominant markers. The co-dominant markers provided sufficient anchors to enable integration of the parental maps. The total map length equals 445 cM. This makes an average of 18 markers per linkage group, the range being from two to 36. The average distance between two markers was 2.2 cM. No gaps larger than 20 cM between two adjacent markers were observed.

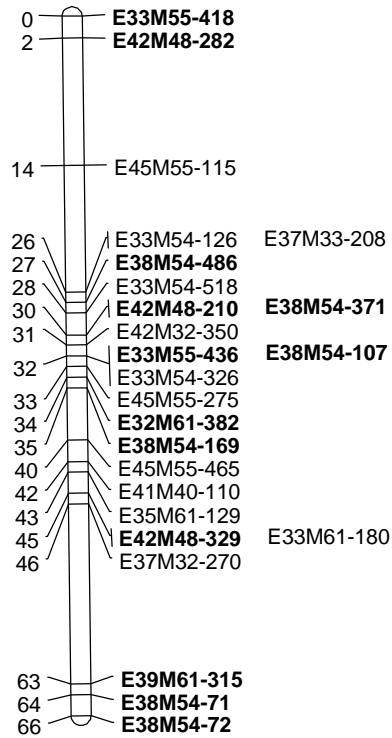
The assignment of linkage groups to barley chromosomes is based on AFLPs and SSRs that are in common with other linkage maps of cultivated barley (see Table 2). Seven of the eleven linkage groups could unambiguously be assigned to known barley chromosomes. Three (groups 2A, U1 and U2) did not contain any common markers and one (group 6) contained markers that mapped to different chromosomes in other mapping populations. Groups 2A and 6 were tentatively assigned to chromosomes based on weak linkage of some markers that were eventually not mapped in our population, but have been mapped in at least one of the other populations.

Figure 1. The linkage map of wild barley, *Hordeum spontaneum*. Assignment of linkage groups to barley chromosomes 1H to 7H as described in the text. Linkage groups U1 and U2 are unassigned. AFLP marker identifiers are composed of primer combinations and estimated length of the amplification product. Co-dominant markers are indicated in boldface, and markers used in identification of chromosomes are indicated in italics. Clusters of markers mapping to the same position (within 1 cM) are indicated by vertical bars to the left of clusters.

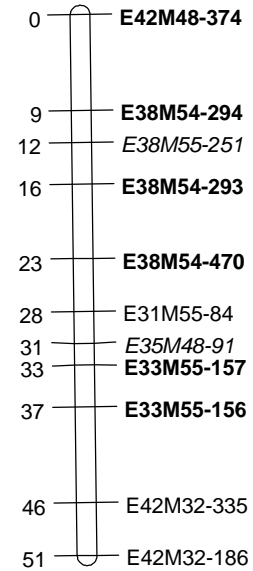
1



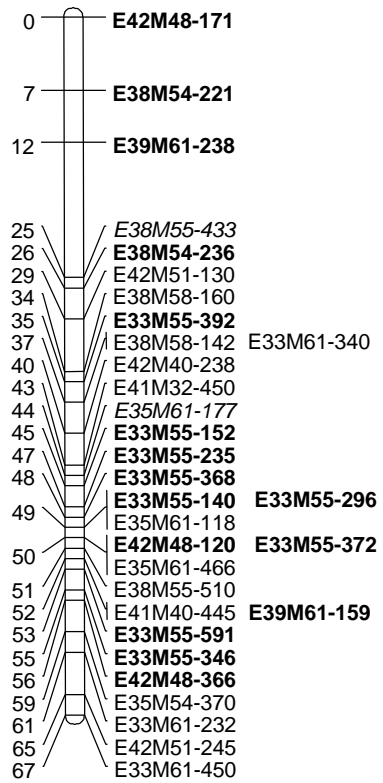
2A



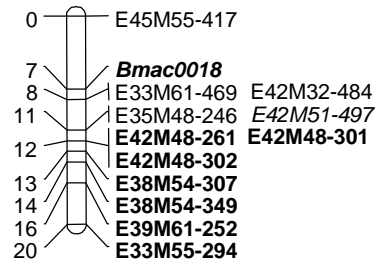
2B



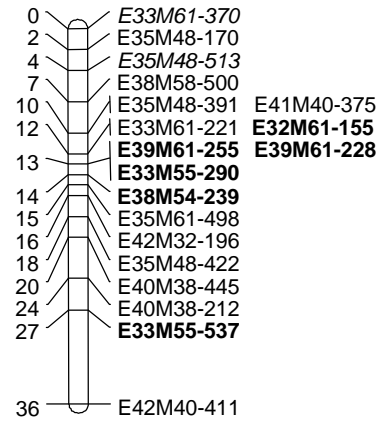
5



6



7



U1

U2

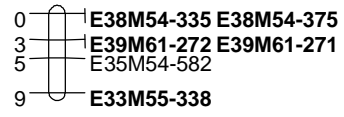
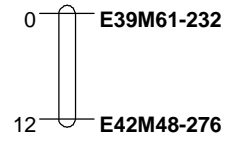


Table 2. The linkage groups, respective chromosomes, number of markers in linkage group, length of linkage group, the maps in which the chromosomes were based on and common markers linking the map of 'Ashkelon' x 'Mehola' to the other maps.

Linkage group	<i>Hordeum</i> chromosome	No. of markers	Length (cM)	Assignment based on	No. of common markers
1	1 (7H)	28	45	L94 x Vada ¹	1
2A	2 (2H ⁶)	25	66		see text
2B	2 (2H)	11	51	L94 x Vada ¹ L94 x 116-5 ²	2
3A	3 (3H)	36	72	Lina x HS ³ L94 x Vada ¹	4
3B	3 (3H)	6	24	Lina x HS ³	1
4	4 (4H)	24	43	L94 x Vada ¹ Proctor x Nudinka ⁴	2
5	5 (1H)	32	67	L94 x Vada ¹	2
6	6 (6H ⁶)	13	20	Lina x HS ³ L94 x Vada ¹ Prisma x Apex ⁵	see text
7	7 (5H)	19	36	L94 x Vada ¹	2
U1	--	2	12	--	--
U2	--	6	9	--	--
Total		202	445		14

¹ Qi *et al.* 1998; ² P. Lindhout, pers. comm.; ³ Ramsay *et al.* 2000; ⁴ Becker *et al.* 1995; ⁵ Yin *et al.* 1999; ⁶ Tentative assignment (see text)
Linkage groups U1 and U2 remained 'unassigned'.

The chi-square values for goodness-of-fit ranged from 0.83 to 1.60 for the 11 linkage groups, indicating a good overall fit. Thus, even though the current map had to be assembled by removing a substantial part of the data, the remaining data still resulted in a reliable map that can serve as a basis for further linkage mapping and QTL mapping in wild barley.

The linkage map presented in this chapter covers only part of the wild barley genome. Although the total number of markers we used should, in principle, be sufficient to cover the genome, the unforeseen high degree of heterozygosity of one of the parents has

hampered the coverage we initially aimed at.

We have estimated the proportion of the wild barley genome covered by the present map in the following way. By repeated sampling of markers, without replacement, from the map we calculated the average map length covered by a marker sample of given size. Average coverage was based on 20,000 samples of given size. By increasing the sample size up to the actual number of markers in the map and by fitting an exponential curve to the relation between sample size and map length covered, we estimated the asymptotic upper limit to be

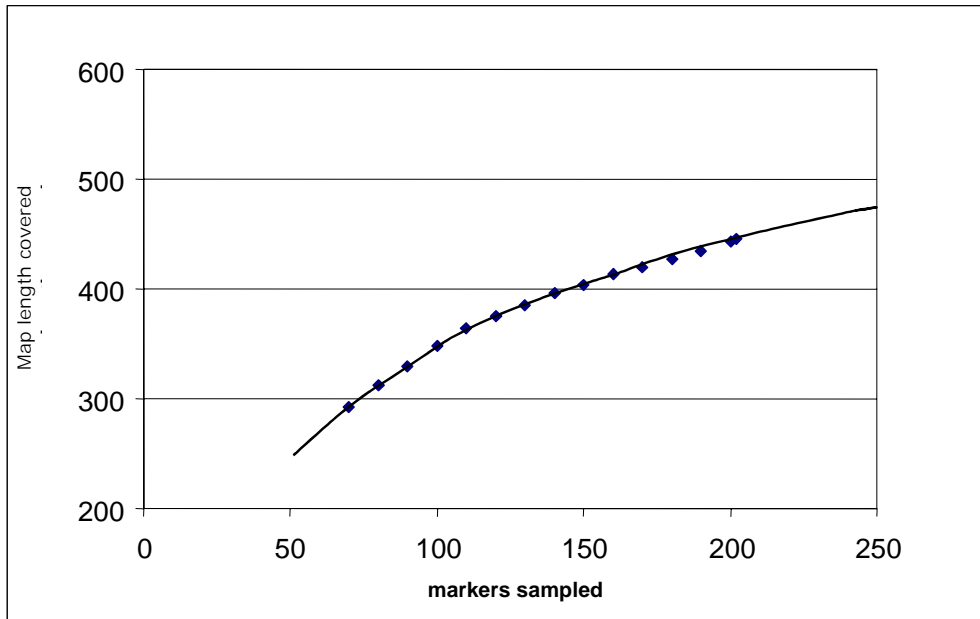


Figure 2. Map length covered by markers randomly sampled from the map. Fitting an exponential curve to the observations yields the estimate of the upper limit, 550 cM.

approximately 550 centimorgans (see Figure 2; the validity of this procedure has been verified extensively using simulated mapping data; Stam, unpublished results.) Therefore, we estimate the total genome length that can be mapped with this population to be about 550 centimorgans. Of this 'mappable' part we have covered a proportion of $445 / 550 = 81\%$. Assuming that the actual total map length of wild barley equals that of cultivated barley, *i.e.* about 1000 centimorgans (*e.g.* Kleinhofs *et al.* 1993, Qi *et al.* 1998, Yin *et al.* 1999, Ramsay *et al.* 2000), we infer that approximately $550 / 1000 = 55\%$ of the wild barley genome can be mapped with this population.

The heterozygosity of the

'Ashkelon' parent is most likely due to a recent outcrossing event, which is known to occur in wild barley (Brown *et al.* 1978a). Because about 45% of the markers could not be mapped, this suggests that altogether the heterozygosity in both parents covers some 45% of the genome. The following observations indicate that the outcrossing must have occurred quite recently (in terms of generations). First, heterozygosity declines rapidly in a predominantly self-fertilising population and it could not be maintained at 45%. Secondly, the heterozygosity cannot be distributed over tiny linkage blocks, dispersed throughout the genome. In that case the 'mappable' markers surrounding such small genomic segments would still

allow an almost complete coverage of the genome. Since the actual 'mappable' proportion of the genome was estimated to be approximately 55%, we conclude that the 'unmappable' part must be concentrated in fairly large linkage blocks, rather than being dispersed over tiny segments. This in turn is in agreement with a recent outcrossing event, because heterozygosity of large genomic segments would rapidly break down into small segments over generations. Thus we conclude that our linkage map has fairly large gaps that cannot readily be filled by additional markers.

Although seven linkage groups could unambiguously be assigned to barley chromosomes, the number of markers shared between the current map and other linkage maps of barley is too

small to allow a detailed comparison of this wild barley map and maps of cultivated barley. Nevertheless, for the purpose of QTL mapping the present map enables a comparison of chromosomal locations of QTLs (Elberse 2002, Poorter *et al.* 2004, Verhoeven *et al.* 2004b).

Acknowledgements

We wish to thank Peter van Tienderen for his stimulating discussions. The fruitful co-operation with Keygene B.V is gratefully acknowledged. The Earth and Life Science Foundation (ALW) which is subsidised by the Netherlands' Organisation for Scientific Research (NWO), financially supported this study.

Chapter 3

Noo, we turn tae 'John Barleycorn. Thou king o' grain.'
It's the unique growin soil, an' the ancient still
An' the generations o' skilled craftsmen
That mak Scotch whiskey the 'soul o' plays an pranks'
Add tae that the pure, peaty, heilan water
An' ye hiv the recipe fur the barley-bree
Tae sample at yur will an 'forget yur loves or debts'.
Shame! The secret o' Whiskey is oot!

H Marshall

(<http://www.it-serve.co.uk/poetry/Worst/secrets.php>)



Environmental, phenotypic and genetic variation of wild barley (*Hordeum spontaneum*) from Israel

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Euphytica, *in press*

Abstract

Wild relatives of crop plants offer an attractive gene pool for cultivar improvement. We evaluated genetic and phenotypic variation for a set of 72 Israeli accessions of wild barley from 21 populations. These populations were grouped further into four ecotypes. In addition, environmental variables describing the local conditions for the populations were used to infer the environmental divergence. Genetic, phenotypic and environmental distances were estimated from the data and UPGMA dendrograms constructed. The results showed that genetic variation was larger between populations than within them, whereas for phenotypic measurements variation was larger within populations than between them. No significant correlation was found between genetic and phenotypic similarities, or between phenotypic and environmental similarities, whereas a weak correlation between genetic and environmental similarities was detected. Twenty-three AFLP markers were identified to be ecotype-specific. Chromosomal location was known for five of these markers. Four of the five ecotype-specific markers were correlated with both phenotypic traits and environmental variables.

Introduction

Wild barley, *Hordeum spontaneum* (C.Koch) Thell. is a progenitor of cultivated barley (*Hordeum vulgare* L.) (Harlan & Zohary 1966). It is a diploid and mostly inbreeding annual, which grows in a wide range of habitats in the eastern Mediterranean and in Southwest Asia. The majority of the primary and

secondary habitats are found in Israel (Nevo 1992). The level of genetic variation of *H. spontaneum* in Israel has been estimated using phenotypic characters, isozyme loci, and different PCR-based molecular markers (*e.g.* Brown *et al.* 1978b; Nevo *et al.* 1979, 1984, 1998; Baum *et al.* 1997; Pakniyat *et al.* 1997; Turpeinen *et al.* 2001). All these studies concluded that genetic

variation in wild barley is substantial. This large genetic variation of wild barley and the fact that it can be crossed with cultivated barley to produce fully fertile hybrids make it attractive for cultivar improvement.

There are several molecular tools for estimating genetic variation. AFLP is a method that has become widely applied in plant population genetics since its development in 1995 (Vos *et al.* 1995). Compared to other methods, the major advantages of AFLP lie in its high diversity index and the number of markers per assay unit (Russell *et al.* 1997). In addition, AFLP markers are reproducible and exhibit intraspecific homology (Roupe van der Voort *et al.* 1997).

Phenotypic characters can also be used to estimate the variation within and between populations. Studies on the correlation between molecular marker and phenotypic distance measures have revealed weak or no association (Pigliucci *et al.* 1991; Burstin & Charcosset 1997; Schut *et al.* 1997; Autrique *et al.* 1996; Ayele *et al.* 1999; Palacios *et al.* 1999). There are several explanations for these observations. One of them is that phenotypic data sets are usually limited in number of traits measured and these

traits are more or less directly influenced by the environment, while molecular markers are potentially numerous and are influenced only indirectly by environment, if at all (Ayele *et al.* 1999).

In this study we have estimated the genetic variation in wild barley populations from Israel using AFLP markers. Variation was also estimated from phenotypic data as well as environmental variables from the same populations. For each type of characterisation the pattern of variation was studied to see whether these differ among the populations. Subsequently, the correlations between the various characterisations were studied in order to detect any general adaptations of the populations to their environment. Finally, associations were studied between individual markers, phenotypic traits and environmental variables. This was done to find out whether there are any correlations between markers from a specific part of chromosome and phenotype or environment.

Table 1. Population and ecotype abbreviations and names, number of accessions per population and ecotype, and AFLP band frequencies as percentages for 21 populations of *H. spontaneum* from Israel.

Ecotype and population abbreviation	Ecotype and population	Number of accessions	Band frequency (%) per ecotype and population
C	Coastal Plain	17	26
AZ	Akhziv	2	1
AT	Atlit	3	40
CA	Caesarea	4	25
HZ	Herzliyya	4	19
AQ	Ashqelon	4	34
M	Mediterranean	18	32
MM	Mount Meron	4	4
MA	Maalot	4	3
SH	Shechem	3	24
BG	Bar-Giyora	4	23
NON	Nahal Oren North	3	28
S	Steppic and Marginal Mediterranean	22	32
MH	Mount Hermon	3	30
TA	Tabigha	3	38
TAB	Tabigha basalt	3	17
TATR	Tabigha terra rossa	3	17
ME	Mehola	3	29
TS	Tel Shoket	4	51
NOS	Nahal Oren South	3	32
D	Desert	15	36
WQ	Wadi Qilt	3	34
SB	Sede Boqer	4	39
RV	Revivim	4	37
YH	Yeroham	4	14

Table 2. Phenotypic traits and their units measured in 21 populations of *H. spontaneum* from Israel (Van Rijn *et al.* 2000).

Type of measurement	Trait	Unit
Biomass allocation related	Water content of leaf	g/g
	Water content of stem	g/g
	Water content of root	g/g
	Leaf area ratio	m ² /kg
	Specific leaf area	m ² /kg
	Leaf mass fraction	g/g
	Stem mass fraction	g/g
Physiological	Root mass fraction	g/g
	Relative growth rate	mg/g/day
	Percentage of respiration	%
	Photosynthesis per unit leaf area	μmol/m ² /s
	Photosynthesis per unit leaf mass	nmol/g/s
	Shoot respiration	nmol/g/s
Chemical	Root respiration	nmol/g/s
	Chlorophyll	μmol/m ²
	Osmotic potential	MPa
	Mineral concentration	mg/g
	Organic acid concentration	mg/g
	Nitrogen concentration	mg/g
	Carbon concentration	mg/g
	Nitrate concentration	mg/g
Morphological	Organic nitrogen	mg/g
	C/N ratio	g/g
	Seed mass	mg
	Height	cm
	Leaf angle	°
	Leaf thickness	μm
	Leaf width	mm
Thickness of epidermis	μm	
	Number of tillers	

Materials and Methods

Accessions

Twenty-one wild barley (*Hordeum spontaneum* (C.Koch) Thell.) populations, containing two to four accessions each (Table 1), were used in this study. The populations were collected from 18 locations in Israel between 1974 and 1978 (Nevo *et al.* 1979, Figure 1). Based on ecological and geographical data, Van Rijn *et al.* (2000) grouped these populations into four ecotypes (Table 1).

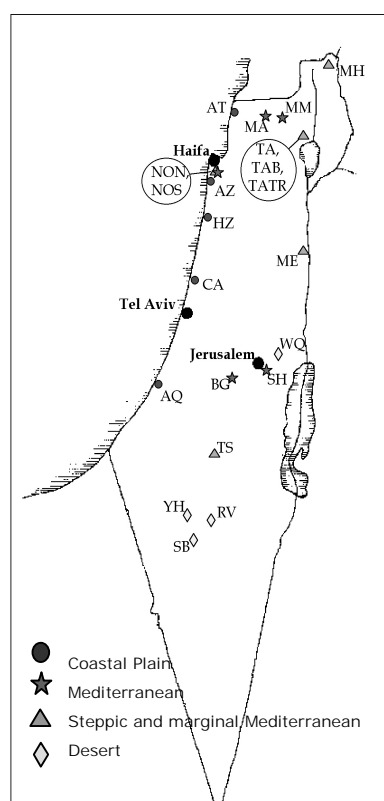


Figure 1. Map of Israel with geographical locations of 21 populations of *H. spontaneum*. For population abbreviations see Table 1.

DNA isolation and AFLP method

Four to ten plants were grown per accession for DNA isolation. Young leaves were collected from two-week old plants. The samples were stored at -80°C . DNA was isolated from 200-300 mg of frozen leaves according to the CTAB protocol (Ausubel *et al.* 1999).

An AFLP protocol was used as described in Vos *et al.* (1995). DNA was digested with restriction enzymes EcoRI and MseI. AFLP bands were visualised by radioactive [$\gamma^{33}\text{P}$]-ATP labelling. Scoring was done by eye, and all clear bands with lengths between 80 and 500 nucleotides were included. In total 11 primer combinations previously tested with cultivated barley (Qi & Lindhout 1997) were used: E32M61, E33M55, E33M58, E35M48, E35M55, E35M61, E38M54, E38M55, E39M61, E42M51, and E45M55.

Phenotypic measurements

Phenotypic data were obtained from a study by Van Rijn *et al.* (2000) in which a total of 30 traits were measured for 72 accessions, including physiological, biomass allocation -related, chemical and morphological characters (Table 2). Plants were grown and measured under close-to-optimal conditions in a growth chamber.

Environmental data

Environmental data were obtained from Nevo *et al.* (1985). It included geographical, temperature, rainfall and humidity data (Table 3).

Table 3. Environmental data for 21 populations of *H. spontaneum* from Israel. Geographical data include longitude east (Long. E), latitude north (Lat. N) and altitude (Alt.); temperature measures include mean annual temperature (Tm), mean temperature in August (Ta), mean temperature in January (Tj), mean seasonal temperature difference (Td), mean day-night temperature difference (Tdd), mean number of hot and dry days (Sh) and mean number of tropical days (Trd); rainfall measurements include mean annual rainfall (Rn) and mean number of rainy days (Rd); humidity measures include mean humidity at 14:00 hours (Hu14), mean annual humidity (Hua), mean number of dewy nights in summer (Dw), Thornthwaite's moisture index (Th) and mean annual evaporation (Ev). These environmental data are from Nevo et al. (1985).

Population abbr.	Geography			Temperature							Rainfall		Humidity		Dw #	Th	Ev
	Long E	Lat. N	Alt (m)	Tm (°C)	Ta (°C)	Tj (°C)	Td (°C)	Tdd (°C)	Sh #	Trd #	Rn (mm)	Rd #	Hu 14	Hua			
AZ	35.10	33.05	10	20	26	12	13	10	30	15	620	56	60	67	59	10	130
AT	34.95	32.70	50	20	26	13	13	9	30	0	500	48	65	72	75	10	133
CA	34.90	32.50	10	20	26	13	13	9	22	10	539	48	65	72	75	10	130
HZ	34.80	32.17	25	20	26	13	13	10	20	0	530	50	65	72	75	30	130
AQ	34.60	31.63	50	20	27	14	13	10	20	80	424	38	64	72	55	30	120
MM	35.40	33.05	1150	14	22	6	16	8	85	0	1010	65	49	61	50	50	155
MA	35.27	33.00	500	17	23	8	15	10	68	0	785	55	50	64	55	10	150
SH	35.23	32.23	400	18	24	9	15	10	70	0	618	45	46	60	42	10	160
BG	35.08	31.72	760	17	26	10	15	9	80	75	537	43	49	61	58	10	155
NON	35.02	32.43	75	19	24	11	--	--	--	--	690	--	59	--	--	--	--
MH	35.75	33.28	1530	11	20	1	19	6	80	0	1600	70	52	58	60	no	160
TA	35.53	32.90	0	24	32	15	17	10	60	120	436	45	45	57	58	30	160
TAB	35.53	32.90	0	24	32	15	17	10	60	120	436	45	45	57	58	30	160
TATR	35.53	32.90	0	24	32	15	17	10	60	120	436	45	45	57	58	30	160
ME	35.48	32.13	-150	22	30	13	17	13	68	183	270	39	34	53	22	50	180
TS	34.92	31.32	375	19	26	11	15	12	60	100	280	32	45	58	55	50	163
NOS	35.02	32.43	75	19	24	11	--	--	--	--	690	--	59	--	--	--	--
WQ	35.38	31.83	50	23	30	14	16	13	110	125	170	32	40	55	25	50	180
SB	34.78	30.87	450	19	26	9	15	13	--	100	91	15	36	53	70	50	168
RV	34.75	31.02	320	20	27	10	15	14	--	130	130	18	38	55	65	50	170
YH	34.90	30.98	490	19	26	10	16	13	--	100	130	18	35	53	65	50	168
Average	35.14	32.24	293	19	26	11	15	10	58	67	520	42	50	61	57	31	154

Statistical methods

Genetic distances were calculated from the AFLP data following the Jaccard distance (Gordon 1981). Phenotypic distances were calculated as Euclidean distances and environmental distances were calculated as simple matching (Gordon 1981). Phenotypic and environmental data were standardised using normal distribution to have mean zero and a standard deviation of 1 ($z=(x-\mu)/\sigma$, where x is the observed measurement, μ is the estimated mean of the trait and σ is the estimated standard deviation of the trait). Longitude and latitude were left out from the distance calculations. These distance calculations were performed with the program PhylTools (Buntjer 2001).

For the genetic, phenotypic and environmental distances, an average linkage, or unweighted pair-group method using arithmetic averages (UPGMA) dendrogram (Nei & Kumar 2000) was constructed. Bootstrapping ($n=100$) was performed to evaluate the robustness of the branching points using Phyltools (Buntjer 2001). The Neighbor and Consense programs from the Phylip (Felsenstein 1993) program package were used to construct the dendrograms that were subsequently visualised using Treeview (Page 1996). Bootstrap values, as percentages of how many times a specific branching point occurred among the 100 resampled trees, from a consensus tree were added into the original tree provided the branching point was the same and the value $>50\%$.

As a formal addition to the graphical comparison of genetic, phenotypic and environmental similarities in the form of dendrograms, Mantel tests were carried out to produce correlations and corresponding permutation tail-probabilities (Manly 1997).

Simple band frequency was used as a measure of gene diversity for AFLP data. The closer to 0.5, the higher the diversity. For ecotype gene diversity measures, all the accessions within the populations of the respective ecotypes were pooled and band frequency estimated. AFLP markers were defined as specific for an ecotype, when band presence/absence was above 0.60 in one ecotype, while below 0.40 in other ecotypes.

Pearson correlations were calculated between previously mapped AFLP markers and phenotypic measurements. These markers were mapped in a wild barley (Chapter 2) or cultivated barley (Qi *et al.*, 1998) mapping populations. As AFLP-markers are biallelic the Pearson correlation coefficient is the equivalent of a t-test on the phenotypic trait using the presence or absence of the marker as classification criterion.

Results and Discussion

A total of 737 AFLP-markers were scored using 11 primer combinations. Of these 637 (85%) were polymorphic over all accessions. The largest variation was observed within TS (51% band presence), a steppic population in the northern Negev Desert, and the smallest within the AZ (1 % band presence), a northern coastline population (Table 1).

Environment

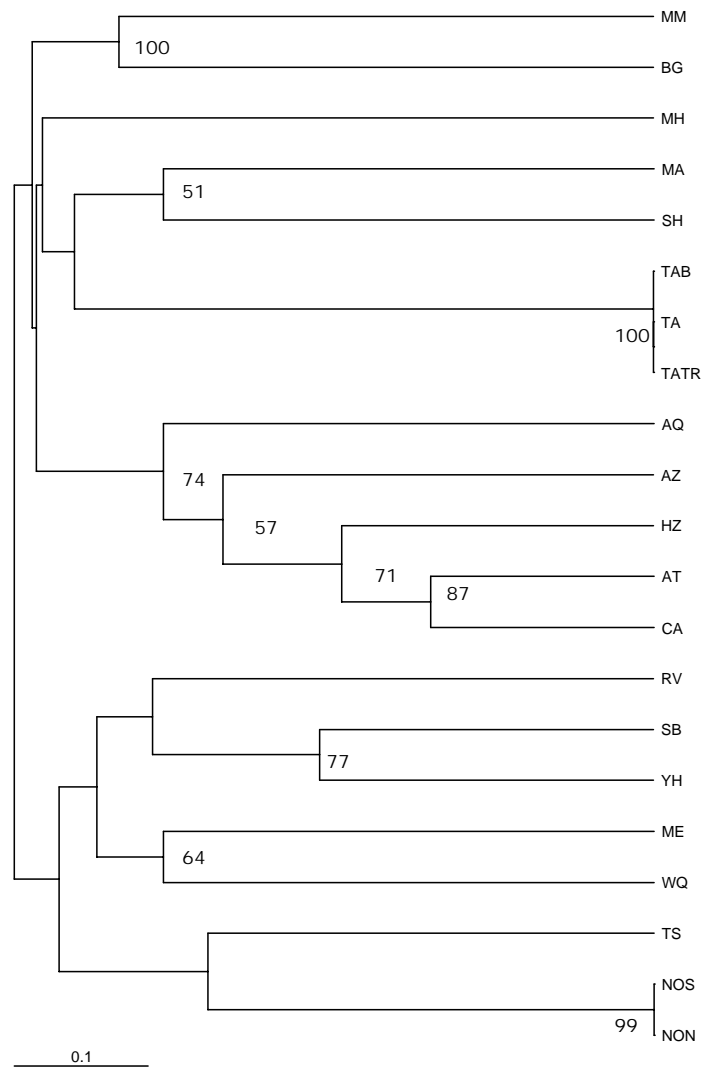
Environment is the directing force that influences the plant populations in the nature. The breeding success of a plant depends on how it can adjust to different environments and grow and reproduce optimally. A plant growing in an environment, which is changing dramatically, whether predictably or not, will need to have more variation genetically to be able to adjust and survive. A stable environment, however, will not put as much pressure on the plant for it to maintain as much genetic variation as the plant living in an unstable environment. Israel is environmentally very diverse and therefore the wild barley populations growing in Israel are of great importance for the possible use in barley breeding.

The populations used in this study were divided into ecotypes by Van Rijn *et al.* (2000). To verify this grouping we calculated simple matching distances from the environmental data and used a dendrogram to visualise the pattern of

variation between populations (Figure 2a). The original choice of ecotypes is reasonably supported by the dendrogram.

The coastal populations form the most distinct cluster in the dendrogram. The populations CA, HZ and AT are located between Tel Aviv and Haifa (*i.e.* geographically close, Figure 1). They form the most robust cluster. The other two coastal populations AZ and AQ are further away (north from Haifa and south from Tel Aviv, respectively) both geographically and environmentally, but still grouping together with the other coastal populations. High humidity, low evaporation and high number of dewy nights are characteristic for the coastal ecotype. There are no altitude differences.

Populations belonging to the Mediterranean ecotype (MA, MM, SH and BG) do not cluster together in the dendrogram. However, they form two pairs: MA and SH form one pair and MM and BG form the other. Although the Mediterranean populations are not geographically close and vary greatly in their altitude, the climate in this ecotype is average compared to the environment of the other populations. If soil type and plant community measures (Nevo *et al.*, 1985) were taken in consideration, these populations clustered together (data not shown). Thus the Mediterranean ecotype is not defined only by altitude, temperature, rainfall and humidity but also by soil type and plant community.



2a.

Figure 2. UPGMA dendrograms of *H. spontaneum* populations and accessions from Israel. Bootstrap values (%) for each branch point are indicated if they are >50%. a) Simple matching distances calculated from environmental data of each population, b) Euclidean distances calculated from phenotypic data of each accession, and c) Jaccard's distances calculated from AFLP markers of each accession. For population abbreviations see Table 1.

The desert populations form their own ecotype, which is very distinct environmentally from the coastal and Mediterranean ecotypes. In fact it is quite the opposite of the coastal ecotype, experiencing the lowest humidity and highest evaporation. The number of dewy days is high, because of the high temperature difference between day and night in the desert.

The least definable populations are the ones belonging to the steppic and marginal Mediterranean ecotype. Based on the environmental data, MH and Tabigha-location -populations cluster among the Mediterranean ecotype. TA has characteristically high temperatures. The steppic populations ME and TS have an environment similar to the desert populations. The two slopes of Nahal Oren cluster together with steppic TS. The environmental data here do not distinguish between the two different slopes; microclimatic data would be needed to do this.

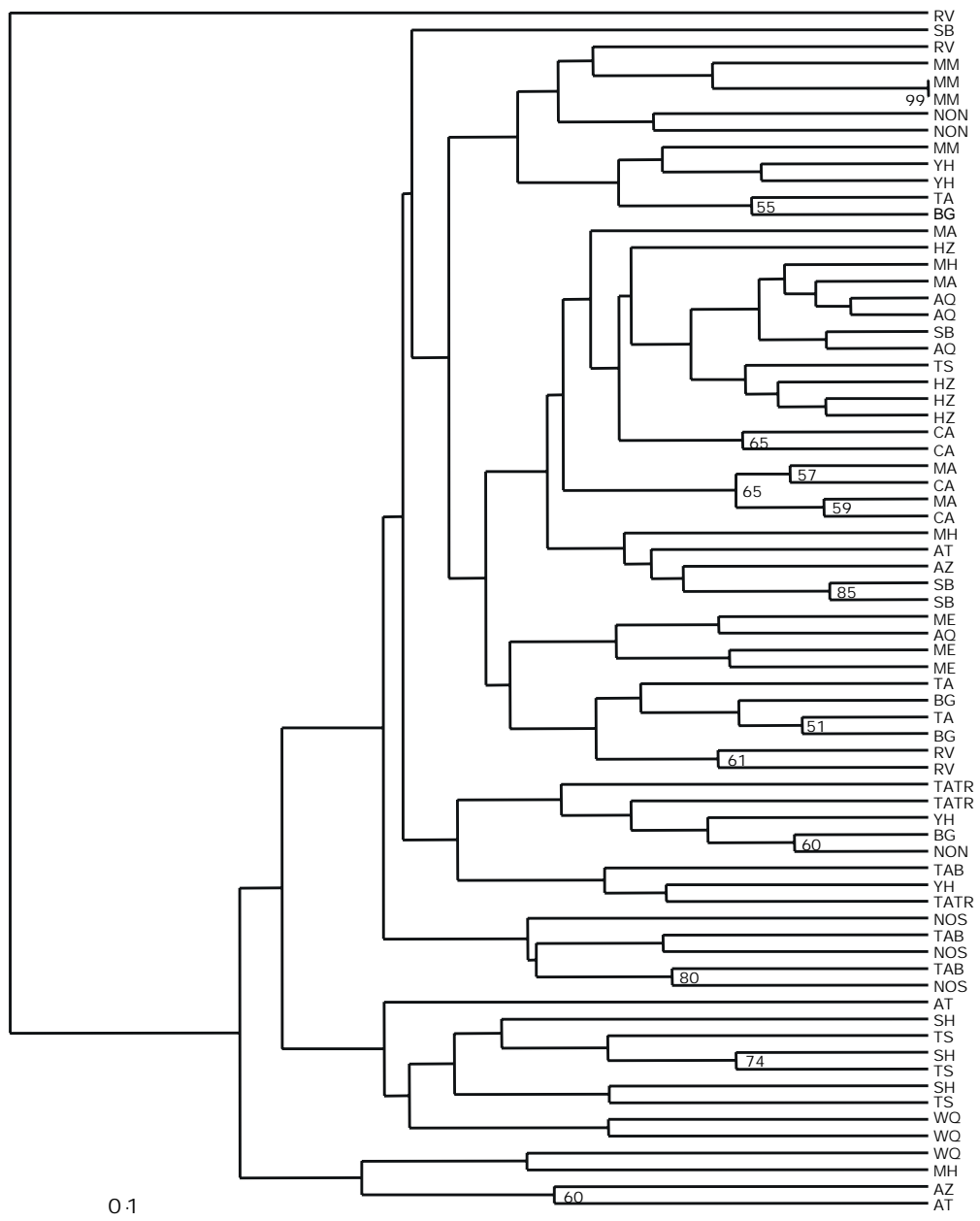
Thus, the wild barley populations in Israel are divided into groups based on their environment. Clear groups are coastal, desert and Mediterranean ecotypes, especially if soil type and plant communities are taken into account, while the fourth ecotype steppic and marginal Mediterranean is only a loose group. If the environment, in the broad sense, has had a strong influence on the phenotypic and genetic variation, the same pattern should emerge from the following results.

Phenotype

Phenotypic traits are the most important target for conventional breeding. Wild species do not always seem to offer phenotypic variation that would improve the qualities of the crop species. However, even if there has not seemingly been useful phenotypic variation for breeding purposes in the wild species, sometimes further research has proven this first observation wrong. A clear example is the introgression of wild tomato alleles to improve the tomato cultivars (Tanksley & McCouch, 1997). Phenotypic variation in wild barley has been studied vigorously and these studies have been concentrating on the traits that would benefit the modern cultivars. This study focuses on the variation found within and among different growth related traits.

Van Rijn *et al.* (2000) initially observed that the phenotypic variation in the traits (except in seed mass, leaf thickness and leaf width) was larger within populations and accessions than between them. These results were further visualised by the dendrogram (Figure 2b) in the present study.

In the dendrogram, the populations do not cluster according to their ecotypes. The phenotypes of the accessions from the Mediterranean population MM cluster closest together. Coastal population HZ clusters mostly together as well, although the bootstrap values are not particularly high. Accessions from the other populations seem to cluster randomly. Overall the



2b.

figure 2 continued

bootstrap values are not very high, which demonstrates again the earlier results of Van Rijn *et al.* (2000). The phenotypic data were measured under close to optimal conditions, and only three morphological traits were more different between populations than within them (Van Rijn *et al.* 2000). This means that under optimal conditions the populations do not differ much from each other and that the population specific adaptations might be disguised.

Genotype

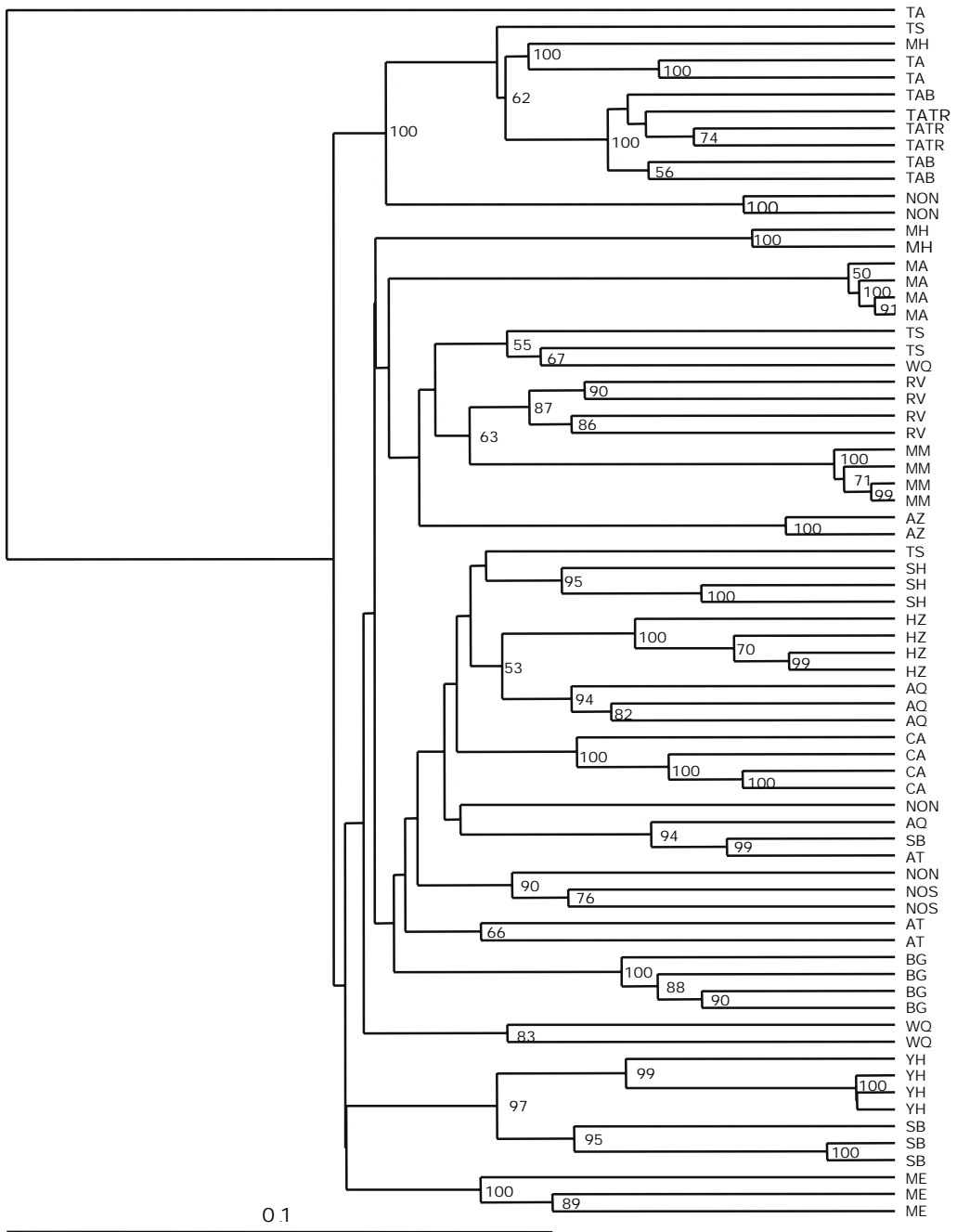
The variation in phenotypic traits is based on the variation and interactions on the genotypic level of the plant as well as on the pressures that the environment places on the plant. Breeding decisions based at least partly on genotypic information offers faster and possibly more durable solutions than conventional breeding through phenotypic selection. However, in order to get to the point where genotypic information can be reliably and efficiently used in breeding, much basic research is needed. Populations from diverse environments such as the wild barley from Israel are very useful for such research, because of the selection pressures the environment imposes upon them. In this paper we have genotyped 72 accessions originally collected from 18 locations in Israel, in order to compare the variation detected with the variation observed in the environment and the phenotype.

In contrast to the phenotype, the genotypic variation was larger

between populations than within them. This was visualised in the dendrogram where accessions were grouping mainly according to their populations (Figure 2c). No distinctly clear groupings of all accessions within their ecotypes occurred. Coastal accessions formed a group with only AZ and AT accessions missing from the ecotype group. This reflects quite well the associations of these coastal populations relative to their environment. The desert populations YH and SB formed also a distinct group as well as the steppic populations from the Tabigha-location including accession from MH and TS. Mediterranean populations MM and MA were the most uniform overall, although coastal population AZ was the least variable according to band frequency.

As expected from the contrasting pattern of variation for phenotypic and genotypic similarities, there was no correlation between these two types of similarity.

The coastal ecotype was in general less variable than the other ecotypes (Table 1). This is also seen from the clustering of the populations within the ecotype in the dendrogram. The distinct environment in the coastal plain might have an influence on this lower genetic variation detected. According to Nevo (2001), the genetic variation is higher in drier and more stressful environments and lower in areas with optimal conditions. Plants do not survive in unpredictable environments without a wider genetic background, whereas in



2c.

figure 2 continued

areas of stable environment they do not have to maintain large genetic variation. In general, the results indicate higher genetic variation in climatically more stressful environments both regionally across Israel and locally at Evolution Canyon (populations NOS and NON). However, even though the coastal ecotype shows the least variation (26%), populations AQ and especially AT have a much higher variation (34 and 40% respectively). When looking at the variation of the populations within each ecotype, the least variable populations are within Mediterranean ecotype, although as an ecotype it is more variable, reflecting the bigger differences between the different populations within it. The coastal population AT is in fact just a bit more variable than the desert populations SB and RV or the steppic TA. This implies that AT has retained its variation in spite of the more optimal climate. However, there are neither microclimatic data from the actual collection sites nor data about the fluctuations of the climate between years. These data would help us to understand what are the real pressures the environment is imposing on the populations.

Many studies have detected associations between individual markers and environmental factors in wild barley (e.g. Nevo *et al.* 1979; Ivandic *et al.* 2002). We have approached this matter from a slightly different angle and looked for ecotype-specific markers. We were able to associate 18 unmapped and five

mapped markers with a certain ecotype (Table 4). Of the five mapped markers two were mapped on chromosome 5 (1H). One of these two (E35M48 433) was associated significantly with several phenotypic data and highly significantly ($p < 0.001$) with the rate of photosynthesis per leaf mass (Van Rijn 2001). The slender desert ecotype, in contrast to the robust Mediterranean ecotype, with significantly lower band frequency of E35M48 433 has on average the thinnest and narrowest leaves, lightest seeds, highest photosynthesis, and fastest relative growth rate (data not shown). High photosynthetic capacity has earlier been associated with dry environments in wild emmer wheat in Israel (Nevo *et al.* 1991). The marker E35M48 433 is also associated highly significantly ($p < 0.001$) with longitude, latitude and mean annual rainfall and significantly ($p < 0.05$) with mean humidity at 14:00 hours (data not shown).

The desert accessions are grouped relatively closely together (except the accessions of WQ), which would account for the association of the marker with the geographical coordinates without altitude (the ecotype does not differ in the altitude). Mean annual rainfall and mean humidity at 14:00 hours were both positively associated with presence of a band in the marker E35M48 433. Thus lower band frequency in the marker E35M48 433 in the desert ecotype mirrors these environmental factors common in deserts

Table 4. Ecotype specific AFLP markers of wild barley, *Hordeum spontaneum*, from Israel.

Marker name	Frequency of present allele				Map position	Association with traits ^c
	C	M	S	D		
E32M61 210	0.65	0.00	0.09	0.33	-	
E32M61 235	0.65	0.11	0.18	0.20	-	
E33M55 230	0.65	0.67	0.27	0.67	chr. 4, 49 cM ^a	oa
E33M55 238	0.18	0.61	0.32	0.20	-	
E33M55 398	0.65	0.33	0.18	0.20	-	
E33M58 394	0.12	0.33	0.27	0.67	-	
E33M58 395	0.82	0.67	0.64	0.33	-	
E33M58 490	0.94	0.89	0.32	1.00	-	
E35M48 246	0.65	0.39	0.95	0.80	chr. 6, 11 cM ^b	lmf
E35M48 433	0.65	1.00	0.73	0.27	chr. 5, 111 cM ^a	d, c, e, lt, m, pa, pm, rgr, lw, sm, a
E35M55 410	0.18	0.61	0.05	0.27	-	
E35M55 87	0.65	0.28	0.91	0.67	-	
E35M61 104	0.24	0.67	0.73	0.80	-	
E35M61 160	0.88	0.67	0.32	0.93	-	
E35M61 216	0.65	0.22	0.36	0.13	-	
E38M54 128	0.35	0.33	0.64	0.13	-	
E38M54 221	0.65	0.61	0.23	0.67	chr. 5, 7 cM ^b	
E38M55 423	0.35	0.28	0.27	0.67	-	
E42M51 101	0.18	0.72	0.18	0.33	chr. 2, 141 cM ^a	m, rmf, smf, wl, ws, lw, sm, a
E42M51 159	0.65	0.33	0.27	0.07	-	
E42M51 325	0.18	0.61	0.18	0.27	-	
E45M55 112	0.88	0.39	0.82	0.67	-	
E45M55 343	0.88	0.67	0.32	0.93	-	
Total	6	7	6	4	5	

^aQi *et al.* 1998 ^bChapter 2 ^cVan Rijn 2001

Their name, frequency in each ecotype, map position (if known) and possible association with traits. Associations with traits were calculated using Pearson correlation coefficient. Traits are oa = organic acids, lmf = leaf mass fraction, rmf = root mass fraction, smf = stem mass fraction, d = density, c = carbon content, e = epidermal thickness, lt = leaf thickness, m = mineral content, pa = photosynthesis per leaf area, pm = photosynthesis per leaf mass, rgr = relative growth rate, lw = leaf width, sm = seed mass, a = leaf angle, wl = water content of leaf, ws = water content of stem.

(low humidity and rainfall) providing a straight link between genotype, phenotype and environment.

In any case, it is not likely that random genetic drift has led to the near fixation of these 23 ecotype specific alleles, because this fixation of alleles is proceeding into the same direction across all the populations within a given

ecotype. These markers may well be associated with genes or QTL, which are in some way advantageous in the given environment. A study to find QTL for drought resistance in wild and cultivated barley cross has been conducted in Israel (Huang *et al.* 2001). It would be interesting to see if there are any correspondences between the QTLs found

and the location of the marker E35M48 437 on chromosome 5.

General conclusions

We have analysed in total 72 accessions from 21 populations of wild barley across Israel both phenotypically and genetically. Including environmental data, we set out to compare the patterns of variation of these accessions and populations in order to detect any general adaptations of the populations to their environment.

The environment differs between groups of populations, where these environmental groups were designated here as ecotypes. The ecotypes do not exhibit special phenotypic features but they do contain ecotype-specific alleles close to fixation. The genotype is furthermore loosely connected to environment, as revealed by clustering of populations to their ecotypes. Multi-marker genotypes do not reflect multi-trait phenotypes (no correlation between genotypic and phenotypic similarities). However, if a specific trait is selected, correlations are found between the trait and specific markers. Also, correlations exist between distances calculated from certain groups of traits, like morphological or physiological traits, and distances calculated from all markers (data not shown). Across selected (mapped) markers and phenotypic traits, the genetic and phenotypic distances exhibited a triangularly shaped relationship (data not shown), *i.e.* when genetic and phenotypic distances were

plotted against each other, genotypically similar accessions were also phenotypically similar whereas genotypically distant accessions were phenotypically either similar or distant, the data points thus forming a triangular shape between the two axes in a diagram. This type of relationship has been observed in other crops as well (Burstin & Charcosset 1997).

Wild barley belongs to the primary gene pool of cultivated barley together with the landraces of barley (Van Hintum 1994). These wild accessions carry an important amount of genetic variation, which is vital for the improvement of modern cultivars with, as a result of domestication and breeding, narrowed genetic backgrounds (*e.g.* Nevo 1992; Nevo 1998). One of the main targets in barley improvement is abiotic stress tolerance to enhance crop reliability (Ellis *et al.* 2000). Before wild accessions can be utilised, they have to be studied in detail. The collection from the Fertile Crescent, where Israel contains the most variable populations (Nevo 1998; Ivandic *et al.* 2002), is an obvious choice for such studies, because it is the centre of diversity as well as the origin of barley domestication (Badr *et al.* 2000). Only Jordan has high genetic diversity of wild barley similar to Israel (Baek *et al.* 2002).

Acknowledgements

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Chapter 4

Let husky wheat the haughs adorn,
An' aits set up their awnie horn,
An' pease and beans at e'en or morn,
Perfume the plain:
Leeze me on thee, John Barleycorn,
Thou king o'grain!

On thee aft Scotland chows her cood,
In souple scones, the wale o'food!
Or tumbling in the boiling flood
Wi' kail an' beef;
But when thou pours thy strong heart's blood,
There thou shines chief.

Robert Burns



Association of AFLP markers with growth-related traits in *Hordeum spontaneum*

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Abstract

*In this chapter, quantitative trait loci (QTL) as identified within a specific cross between two wild barley (*Hordeum spontaneum*) populations are compared with marker-trait associations assessed across a set of 81 wild barley accessions. The accessions were measured for 33 traits related to growth, C and N economy as well as plant morphology. The accessions were genotyped for 68 AFLP markers, mapped earlier in cultivated barley as well as in wild barley. Of the 68 markers 60 had significant correlations to one or more traits. Marker-trait associations supported several QTL detected earlier. For relative growth rate, two out of four QTL were supported. Four markers related to photosynthetic rate per unit leaf area were mapped on chromosome 4, where also the QTL for photosynthetic rate and stomatal conductance were found. One of these markers was also related to chlorophyll content, suggesting that this location on chromosome 4 might be very important for photosynthesis-related traits. One QTL was detected for specific leaf area on chromosome 7. This QTL was supported by the only marker-trait association for specific leaf area. Markers on chromosome 1, 2 and 4, were strongly associated with leaf thickness, leaf width and seed mass, suggesting that these traits are genetically linked. An alternative to the classical Bonferroni correction for controlling the false discovery rate in multiple testing is discussed and applied to the test results. This leads to a less stringent significance threshold for individual tests than the Bonferroni-based correction.*

Introduction

Plant species differ considerably in their potential relative growth rate (RGR, net increase in biomass per unit biomass already present per unit of time) with plant species from fertile habitats showing much higher RGRs under optimal conditions than those from infertile habitats (Grime & Hunt, 1975; Poorter, 1989). These differences in RGR are mainly due to variation in specific leaf area (SLA, leaf area per unit leaf mass) and to a lesser extent due to leaf mass

fraction (LMF, leaf mass per unit of plant mass) (for a review see Poorter & Van de Werf, 1998). A range of physiological, chemical and morphological traits is correlated with the potential RGR of a plant (Lambers & Poorter, 1992).

Wild barley (*H. spontaneum*), the progenitor of cultivated barley is widely distributed in the Fertile Crescent (Zohary & Hopf, 1988) and occupies a wide range of different habitats (Nevo *et al.* 1979). The populations collected from around the Fertile Crescent are genetically divergent (for a review see

Nevo, 1992; Forster 1999). The species also shows large morphological and physiological variation which can be related at least partly to the ecogeographical origin of the populations studied (e.g. Nevo *et al.* 1986b; Forster 2000; Chapter 3).

The variation in physiological and morphological growth traits within and among populations from Israel has been determined under standardised, controlled conditions (Van Rijn *et al.* 2000). Two accessions of these populations, divergent for growth-related traits, were crossed and an AFLP marker map was constructed (Chapter 2). Growth-related traits were measured on the offspring (F₃-lines) and quantitative trait loci (QTL) were detected (Poorter *et al.* 2004).

An alternative way of linking genetic markers to traits is analysis of marker-trait associations in a set of accessions or populations (Pakniyat *et al.* 1997; Forster *et al.* 2000). Using a set of wild barley accessions instead of a mapping population of a cross between two parents has a number of advantages. First, there is no need to develop an experimental cross. Second, the observed associations are not limited to a single cross (Kraakman *et al.* 2000), which enables evaluation of a much wider germplasm. A third, related advantage is that screening of a wider range of genotypes minimises the risk run in QTL experiments that both parents by chance have the same allele for a number of traits (Kraakman *et al.* 2000).

In this study, AFLP markers were associated with the variation in growth traits in *H. spontaneum* populations and compared with the QTL found by Poorter *et al.* (2004).

Material and Methods

Plant material

Eighty-one wild barley accessions grouped in 21 populations from a wide range of locations in Israel, were used in this study. The populations along with available climatic data of the locations are listed in Chapter 3.

Seeds were germinated on moistened filter paper on Petri dishes at 6°C and an irradiance of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After one week seedlings were transferred to a white beach sand filled container with drainage holes. The sand was saturated with half strength of the following nutrient solution: 603 μM $\text{Ca}(\text{NO}_3)_2$, 795 μM KNO_3 , 190 μM KH_2PO_4 , 270 μM MgSO_4 , 0.2 μM MnSO_4 , 0.9 μM ZnSO_4 , 20 μM H_3BO_3 , 0.3 μM Na_2MoO_4 , 40 μM Fe-EDTA, 40 μM FeSO_4 and 47 μM SiO_2 . The container was placed in a growth room for five days in the following conditions: 14/10 h day/night, 20°C day/night, irradiance of $450 \pm 25 \mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity 70%. Thereafter seedlings were transferred to 33 L tanks containing the nutrient solution described above, aerated and at full-strength, which was replaced weekly. The pH of the nutrient

solution was adjusted regularly to 5.8 with H₂SO₄. To avoid mutual shading, the number of plants on each container ranged from 18 and 6, depending on the size of the plants. Plants were rotated four times a week within the growth room.

DNA isolation and AFLP analysis

Four to ten plants were grown per accession for DNA isolation. Leaves were collected from two-week old plants. DNA was isolated from 200-300 mg of frozen (-80°C) leaves according to the CTAB method (Ausubel *et al.* 1999).

The AFLP protocol was used as described in Vos *et al.* (1995). The DNA was digested with restriction enzymes EcoRI and MseI. The AFLP bands were visualised by radioactive [³²P]-ATP labelling. The presence or absence (dominant scoring) of AFLP fragments was scored by eye, and all clear bands with lengths between 80 and 500 nucleotides were included. In total 10 primer combinations previously tested with *H. vulgare* (Qi *et al.* 1997) were used: E32M61, E33M55, E35M48, E35M55, E35M61, E38M54, E38M55, E39M61, E42M51, E45M55. Only markers that were mapped in a cultivated barley cross 'L94 x Vada' (Qi *et al.* 1998) and in a wild barley cross 'Ashqelon x Mehola' (Chapter 2) were included in the analysis.

Experimental design

Plants from four accessions per population were grown. Eight plants per accession were used to measure a range

of traits, somewhat arbitrarily categorised in three groups related to growth analysis, C and N-economy and morphology. Plants were measured at 23 to 25 days after germination, when there were 2-10 tillers. Whole-shoot photosynthesis and shoot and root respiration were measured on two plants of each accession. Fresh and dry mass of leaves, stems and roots, leaf area, leaf width, leaf angle, shoot height and the number of leaves and tillers were also determined on these two plants as well as on two additional plants. Two other plants were used for measurements of osmotic potential and to determine the chlorophyll concentration and the remaining two plants were used for measurement of leaf thickness. The latter four plants were also used for chemical analyses. Because of the large number of plants and the time required for the physiological measurements we staggered the germination and growth of the plants. Four randomly chosen accessions of different populations were measured each week.

Measurements

Seed mass of each seed was determined separately. Leaf width and thickness were measured on the youngest fully expanded leaf. Leaf angle was assessed as the average angle of the four oldest leaves. Plant height was determined by measuring total shoot length.

Leaf area, fresh and dry mass of the leaves (leaf blades), stems (leaf sheaths) and roots were determined to

calculate water content (fresh mass - dry mass / dry mass) of leaf, stem and root (WCleaf, WCstem, WCroot, respectively), leaf area ratio (LAR, leaf area per total plant dry mass), specific leaf area (SLA, leaf area per leaf dry mass), leaf mass fraction (LMF, leaf dry mass per total plant dry mass), stem mass fraction (SMF, stem dry mass per total plant dry mass) and root mass fraction (RMF, root dry mass per total plant dry mass).

Carbon concentration and nitrogen concentration were determined with an elemental analyser (Carlo Erba 1110, Milan, Italy). Nitrate concentration was quantified according to Cataldo *et al.* (1975). Ash and ash alkalinity were determined as described by Poorter and Villar (1997). Results were used to calculate concentrations of organic acids, minerals and organic nitrogen compounds. The osmotic potential of the leaf sap was measured using a Wescor Vapour Pressure Osmometer (Logan, UT, USA). The chlorophyll concentration of the leaf was determined according to Lichtenthaler and Wellburn (1983) after extraction with 80% acetone.

Net photosynthesis, dark respiration and root respiration were measured as CO₂ exchange. CO₂ and H₂O exchange were measured differentially to calculate photosynthesis per unit leaf area and per unit leaf mass, shoot respiration, root respiration and water use efficiency.

Further details on the phenotypic analyses are given in Van Rijn *et al.* (2000).

Statistical analyses

Relative growth rate (RGR) was estimated, on the basis of a plant's C-economy, using the formula given in Poorter & Pothmann (1992):

$$RGR = \frac{PS_a \times SLA \times LMF - ShR \times (LMF + SMF) - RR \times RMF}{C}$$

where, PS_a = daily rate of Photosynthesis per unit leaf area, ShR = daily Shoot Respiration, RR = daily Root Respiration, SLA = Specific Leaf Area, LMF = Leaf Mass Fraction, SMF = Stem Mass Fraction, RMF = Root Mass Fraction and C = Carbon concentration of the plant biomass). We determined the carbon content of the leaves, assuming that it is representative of that of the whole plant. In reality, the carbon content of roots and shoots tend to be slightly lower than in leaves (Poorter & Bergkotte, 1992), but these differences are not likely to affect the RGR-calculation to more than a small extent. A second assumption is that the rates of photosynthesis and respiration, both measured over a two-hour period, can be integrated over 24 hours. All parameters of the RGR-formula were measured on the same day.

To study marker-trait associations, simple product moment correlations were calculated (Pearson correlations) between a selected set of markers and the 33 quantitative traits. Markers were included if they were mapped to either 'Ashqelon x Mehola' (Chapter 2) or the 'L94 x Vada' (Qi *et al.*

1998) map. Of the original 643 polymorphic markers, 68 were mapped. A test for the correlation is in this case equivalent to a two-sample t-test comparing the mean of the accessions with a specific marker band with the mean of the accessions without that marker band. A significance level of 0.05 was chosen, besides a more strict 0.0007 (a Bonferroni corrected level of significance; 0.05 divided by the number of selected markers). All the calculations were performed using SAS (SAS system for Windows 6.12, 1989-1996 SAS Institute Inc. Cary, NC, USA).

Results

As mentioned earlier, of the 643 polymorphic markers 68 were mapped. Of these 68 mapped markers, 60 correlated significantly ($p < 0.05$) with at least one of the measured traits. For these 60 markers in total 267 marker-trait correlations were observed at the 0.05 significance level. 2244 tests were performed and at the 0.05 significance level 112 false positives are expected. At the strict significance level of 0.0007, 32 marker-trait correlations were found significant. Only 1.5 false positives are expected at this level. The strongest correlation was found between seed mass and a marker from chromosome 4 (33 cM in 'Ashqelon x Mehola' map; $r = -0.70$). There were in total 13 marker-trait associations with $p \leq 0.0001$. Ten of these

were associations between several markers and morphological traits, and three between a marker (chromosome 5, 26cM on 'Askelon x Mehola' map) and chemical traits CN, mineral content and N.

Figure 1 shows the results of the marker-trait correlations positioned on the 'L94 x Vada' and 'Ashqelon x Mehola' maps. The QTL are from Poorter *et al.* (2004).

Mapped QTL supported by marker-trait associations

Fifteen QTL were clearly supported by at least one marker-trait correlation at the 0.05 significance level (Table 1). At the strict 0.0007 significance level four marker-trait correlations (on chromosomes 2 and 4) supported four QTL. Supportive marker-trait associations are indicated as grey boxes in Figure 1. Two QTL (height and seed mass) on chromosome 2 were supported by marker-trait association only if the 'Ashkelon x Mehola' chromosome is orientated as shown in Figure 1. The remaining 13 supported QTL were clear in this respect.

Mapped QTL not supported by marker-trait associations

There were 17 QTL that were not clearly supported by the marker-trait correlations. In 11 cases significant marker-trait correlations were detected at the same chromosome as the corresponding QTL, but not in the actual position of the QTL. In one of these cases

the associated marker lies on the edge of the QTL interval (LMF on chromosome 7).

There were several traits that were not measured in the population study (Table 1). However, some of these are strongly correlated with some other traits that were measured in the populations. A good example is the density measures. Leaf mass density (LMD) was estimated within the populations whereas root (RMD) and stem mass density (SMD) were determined in the F_3 . There were three QTL detected for both RMD and SMD. Two of the SMD QTL were supported unambiguously by marker-LMD association. One RMD QTL on chromosome 1 was supported only if the orientation is as shown in Figure 1. Another example of traits not measured in the natural populations is leaf length. Leaf length is strongly correlated with leaf width and all the four QTL are supported

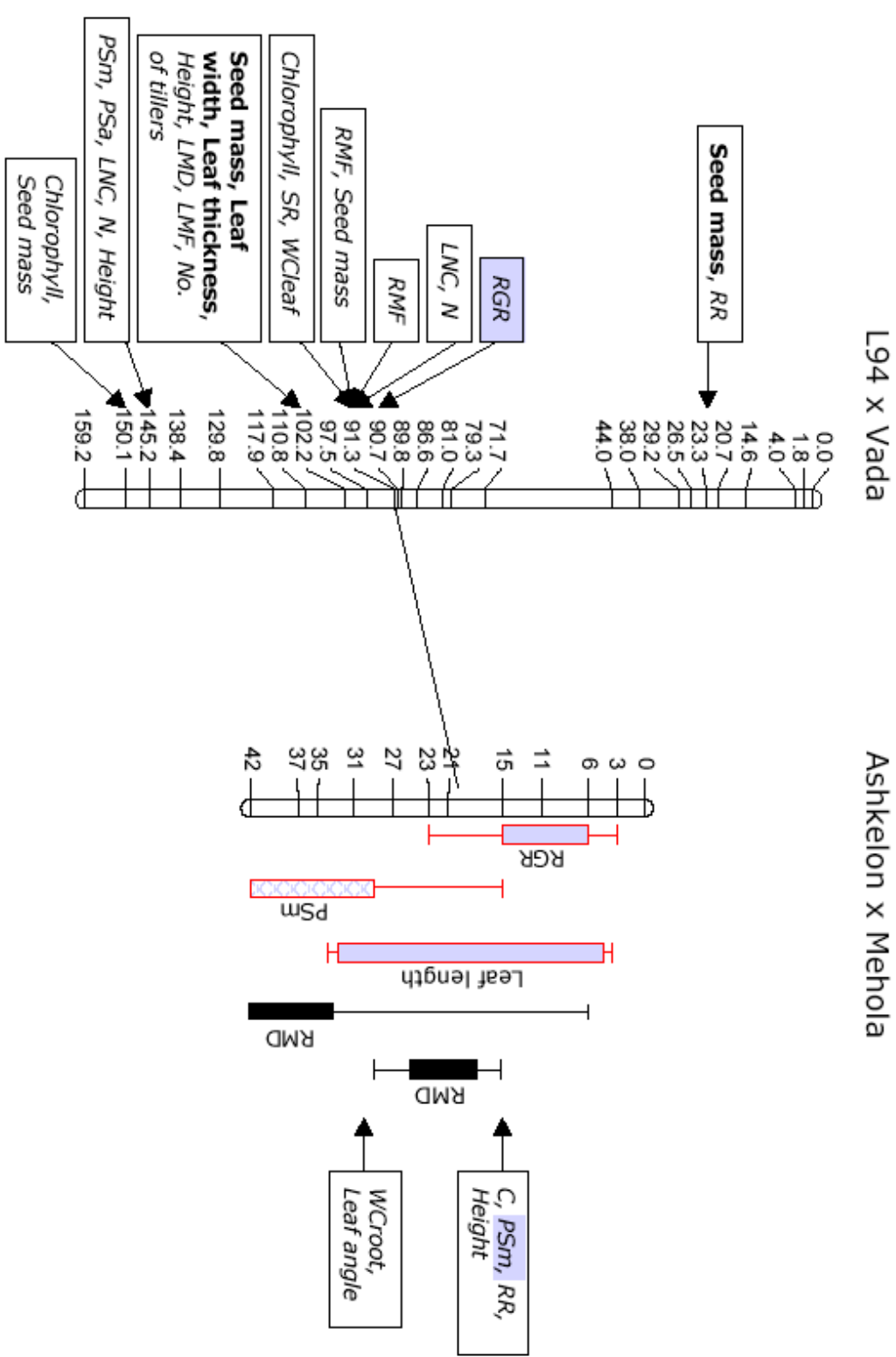
by marker-leaf width associations, three of these at the 0.0007 significance level.

Other marker-trait associations

The strongest correlations with genetic markers were observed for morphological traits such as seed mass and leaf width or thickness. RGR showed also a strong correlation with a marker on chromosome 2 together with one of its components, RMF. On chromosome 5, three chemical traits, mineral content, N and CN, were highly significant as well as the rate of photosynthesis per mass and leaf width. Chromosomes 3, 6 and 7 contained only one or two strong marker-trait correlations whereas chromosomes 2 and 4 contained many highly significant associations.

Figure 1. The significant marker-trait correlations among *H. spontaneum* accessions and QTLs in a *H. spontaneum* cross. The 60 markers were based on two linkage maps; 'L94 x Vada' (Qi *et al.* 1998) and 'Ashqelon x Mehola' (Chapter 2). The arrows point to the marker positions. The marker-trait correlations are shown on the left side of the chromosomes. The traits that correlate with a marker at the 0.0007 significance level are shown in boldface, and those that correlate at the 0.05 significance level are shown in italics. The traits that correlate with a marker and support a QTL are shown in grey boxes. The QTL with 1 and 2 LOD confidence intervals are given on the right side of the 'Ashqelon x Mehola' map. The QTL with positive effect of the Ashkelon allele are shown in black (LOD >3.1), or slanted black (LOD 2.5-3.1) and the QTL with positive effect of the Mehola allele are shown in grey (LOD >3.1) or slanted grey (LOD 2.5-3.1). The common markers shared between the two maps are connected with lines. Dashed lines are used to point to the approximate positions of the SSR markers (Ramsay *et al.* 2000) in the 'L94 x Vada' map.

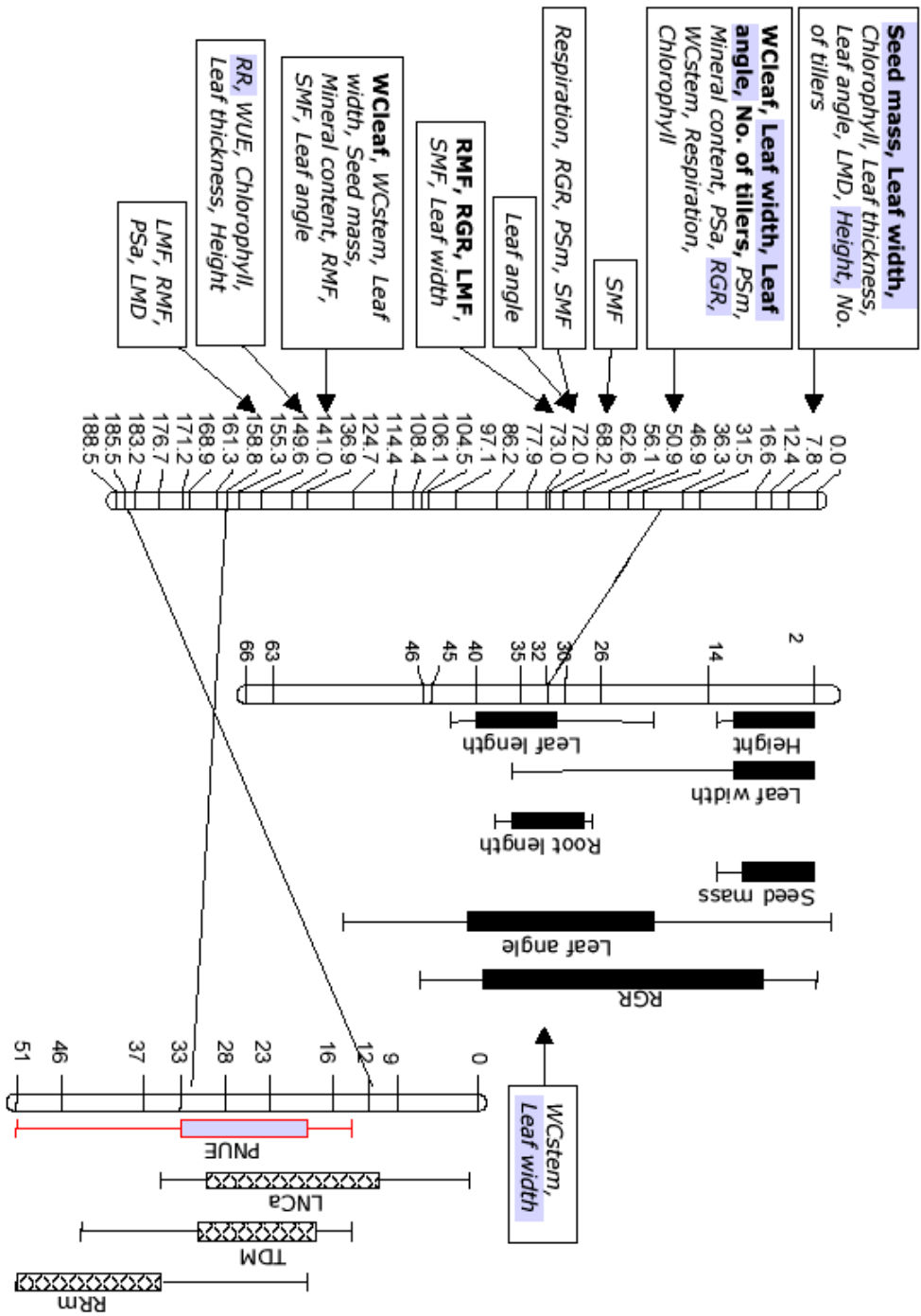
Chromosome 1



Chromosome 2

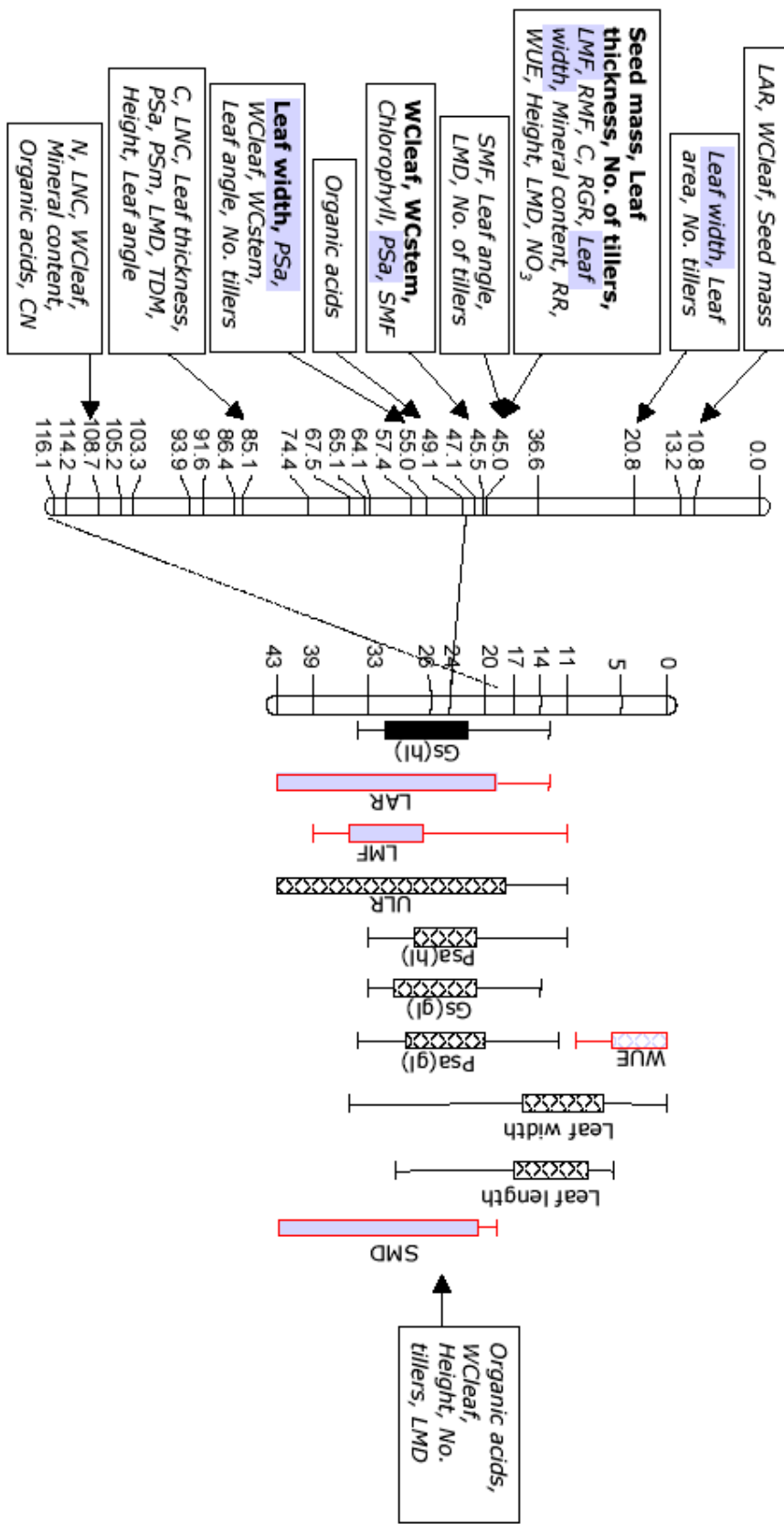
L94 x Vada

Ashkelon x Mehola



Chromosome 4

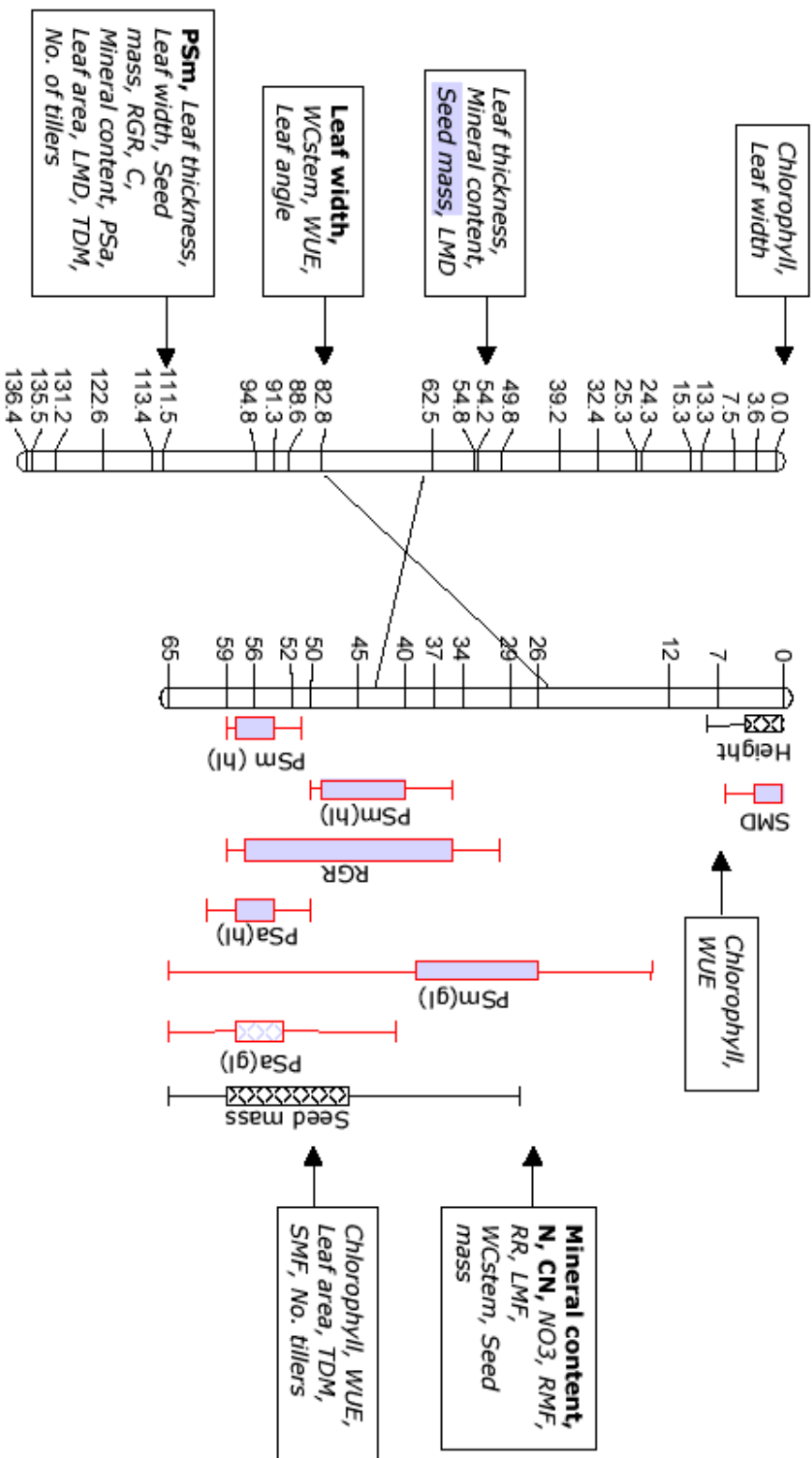
L94 x Vada Ashkelon x Mehola



Chromosome 5

L94 x Vada

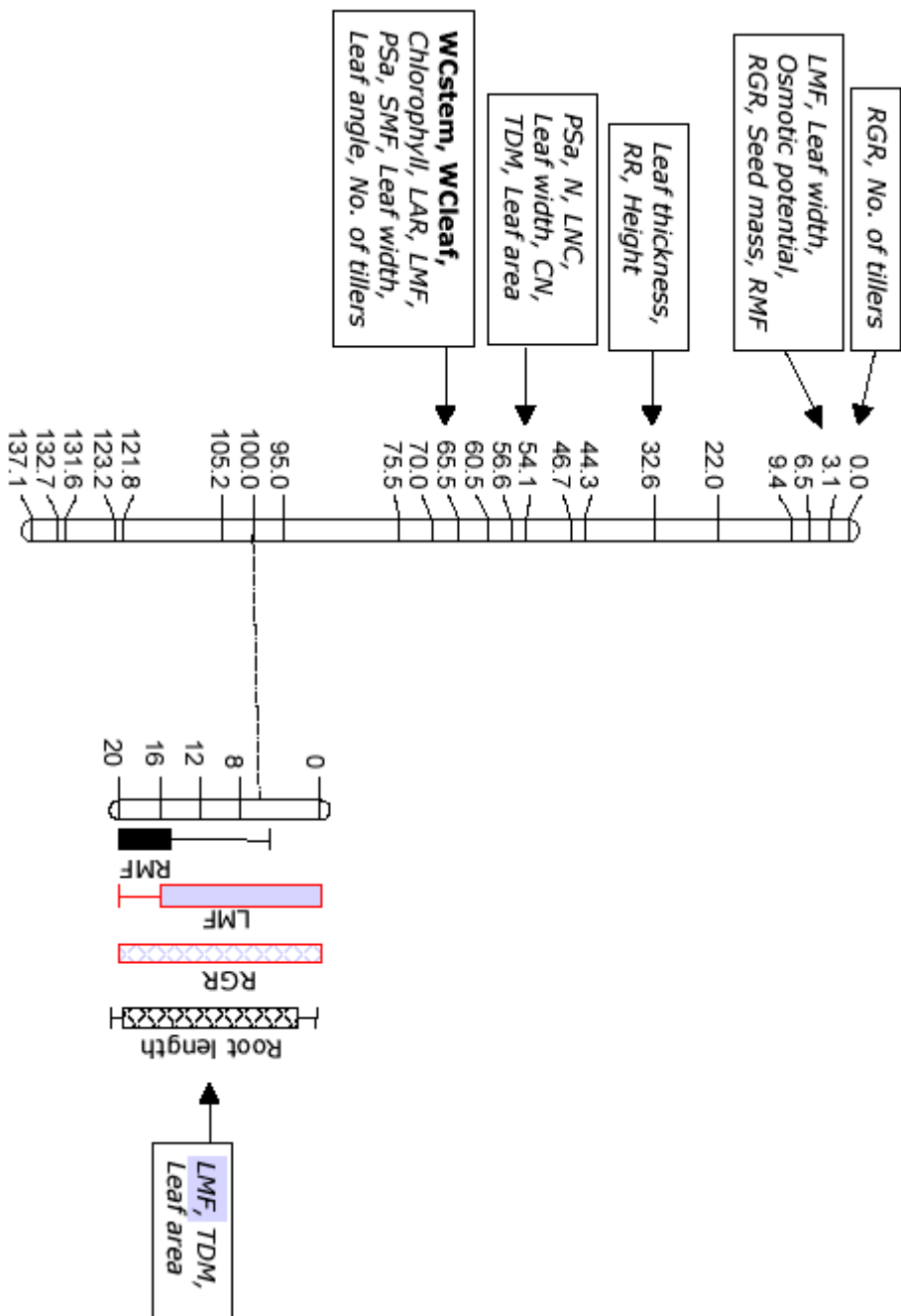
Ashkelon x Mehola



Chromosome 6

L94 x Vada

Ashkelon x Mehola



Chromosome 7

L94 x Vada

Ashkelon x Mehola

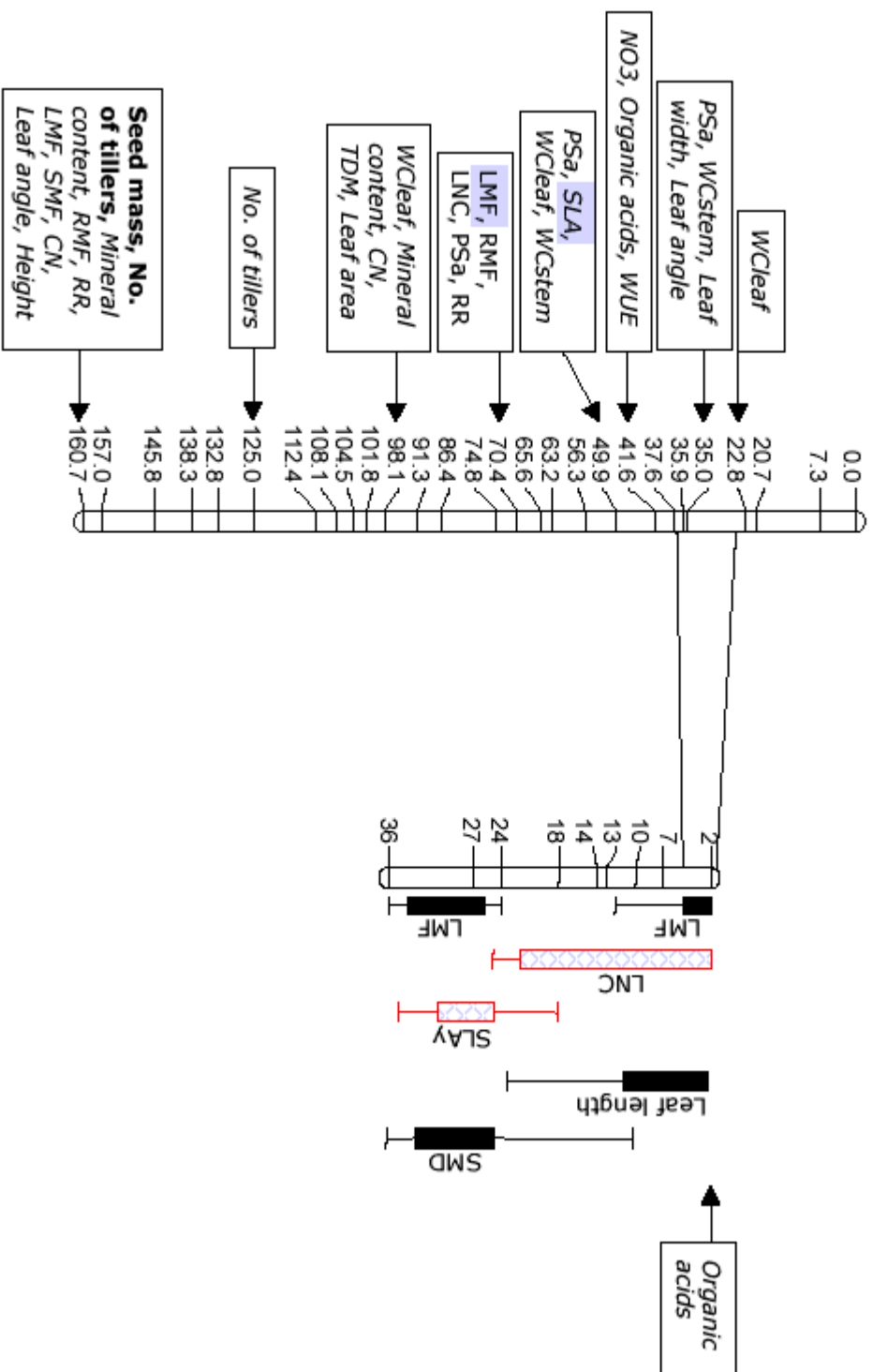


Table 1. The traits measured in wild barley accessions and F₃ of the cross 'Ashkelon x Mehola', the number of marker-trait correlations at 0.05 and 0.0007 significance levels, number of QTL detected by Poorter *et al.* (2004) and the number of supported QTL by one or more marker-trait correlations at the 0.05 and 0.0007 significance levels.

Trait	Number of marker-trait correlations*		QTL	Number of QTL supported*	
	P<0.05	P<0.0007		p<0.05	p<0.0007
<i>Growth related</i>					
Total dry mass (TDM)	6	1	1	0	0
Leaf area ratio (LAR)	2	0	1	0	0
Leaf mass fraction (LMF)	10	1	4	2	0
Relative growth rate (RGR)	8	1	4	2	0
Root mass fraction (RMF)	10	1	1	0	0
Specific leaf area (SLA)	1	0	1	1	0
Stem mass fraction (SMF)	10	0	0	0	0
<i>C and N economy related</i>					
Carbon content (C)	7	0	0	0	0
Chlorophyll	10	0	0	0	0
Mineral content	10	1	0	0	0
Nitrogen content (N)	5	1	0	0	0
NO ₃	6	0	0	0	0
Organic acids	6	0	0	0	0
Organic nitrogen/leaf area (LNC)	8	0	2	0	0
C/N ratio (CN)	6	1	-	-	-
Osmotic potential	1	0	0	0	0
Photosynthesis/leaf area (PS _A)	12	0	4	2	0
Photosynthesis/leaf mass (PS _M)	8	1	4	1	0
%Respiration	2	0	0	0	0
Root respiration (RR)	8	0	1	1	0
Shoot respiration (SR)	1	0	0	0	0
Water use efficiency (WUE)	6	0	1	0	0
<i>Morphological</i>					
Height	12	0	2	1	0
Leaf Angle	11	1	2	1	1
Leaf area	7	0	-	-	-
Leaf mass density (LMD)	10	0	-	-	-
Leaf thickness	10	2	0	0	0
Leaf width	17	6	2	2	2
Number of tillers	14	3	1	0	0
Seed mass	16	5	2	2	1
Water content leaf (WCleaf)	13	4	0	0	0
Water content root (WCroot)	3	1	0	0	0
Water content stem (WCstem)	11	2	0	0	0
<i>Traits not measured in populations</i>					
Leaf length	-	-	4	-	-
Root length	-	-	2	-	-
Photosynthetic nitrogen use	-	-	2	-	-
Stem mass density (SMD)	-	-	3	-	-
Root mass density (RMD)	-	-	3	-	-
Stomatal conductance (Gs)	-	-	2	-	-
Unit leaf rate (ULR)	-	-	1	-	-
Total	267	32	50	15	4

*The numbers at 0.0007 significance level are included in the columns for p<0.05

Multiple testing

One problem with association mapping is the issue of multiple testing when evaluating hundreds of markers for tens of traits. Selecting an appropriate

significance level when multiple tests are performed is a difficult task and the Bonferroni correction is often used to control the type I error. However, this is sometimes overly stringent and many

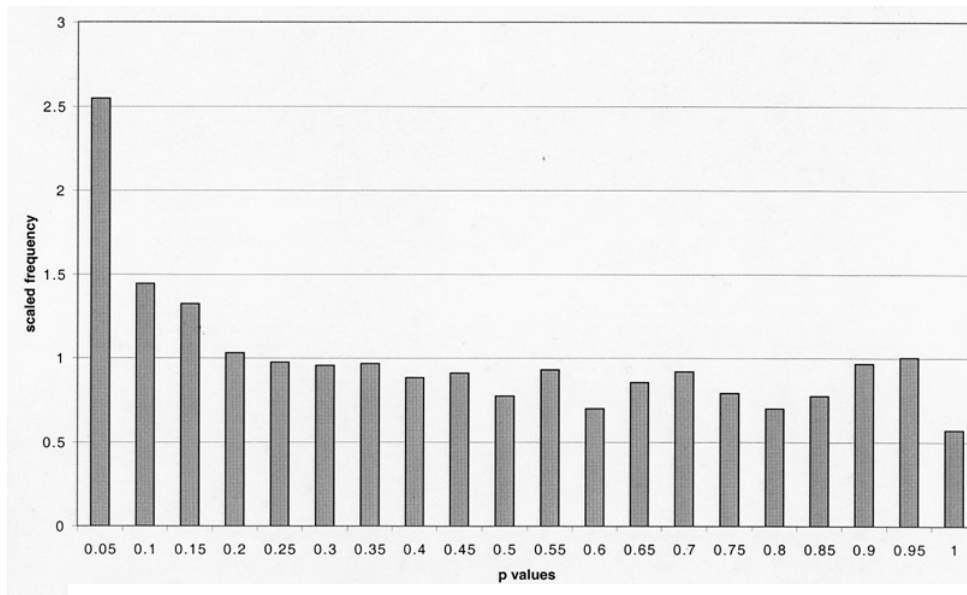


Figure 2. Scaled frequency histogram of p-values of the correlation tests.

true alternative hypotheses are discarded as non-significant. Storey and Tibshirani (2003) suggest another way of determining significance in genomewide studies. The false discovery rate (FDR; originally introduced by Benjamini and Hochberg 1995) is defined as the estimated proportion of the false positives among the tests deemed to be significant. For calculation of FDR, the proportion of true nulls (π_0) among the tests has to be estimated. Applications of FDR to date have assumed π_0 to be one, *i.e.* all tests are a priori assumed to be true nulls. Storey and Tibshirani (2003) estimate π_0 using the distribution of the p-values from all the tests. When all tests are true nulls, a uniform distribution of p-values between zero and one is expected. That is, when in reality none of the

markers have an effect on the trait. Figure 2 presents a scaled frequency histogram of wild barley marker-trait association p-values from the current chapter. This clearly shows that a proportion of the results represents true alternatives, *i.e.* a proportion of the marker-trait correlations are expected to be real. Therefore, the histogram is a mixture of two distributions: a uniform distribution of true nulls and an unknown distribution of true alternatives, mainly concentrated close to zero. π_0 is estimated as the proportion of uniformly distributed p-values in the mixture (Figure 3a). The next step is to determine the FDR, or q-values for the different tests. The q-value for a given test is the minimum false discovery rate incurred by calling that test significant. FDR

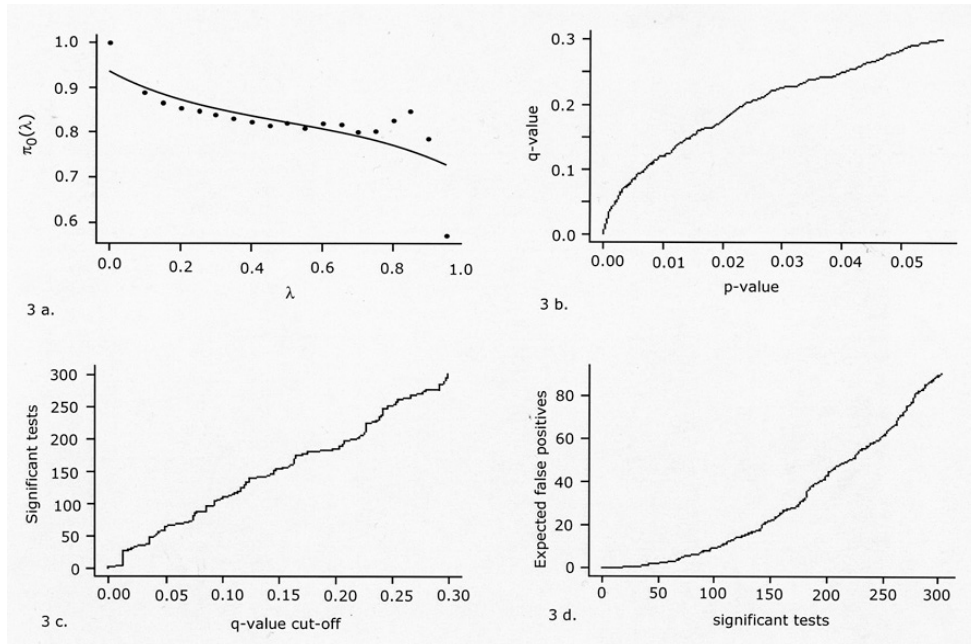


Figure 3. Four graphs illustrating a. the estimation of π_0 (where λ is p-value cut-off and the points represent the proportion of results $p > \lambda$; the estimated π_0 at $\lambda=1$, is 0.729), b. the relationship between q- and p-values, c. q-value cut-off in relation to the number of significant tests and d. the number of expected false positives within the amount of significant tests. The graphs were made in R-script QVALUE v 1.1 (Storey 2002) based on the p-values.

estimation was done with the help of the R-script QVALUE v.1.1 (Storey, 2002). For the association experiment, π_0 was estimated to be 0.73, implicating that 0.27 ($1-\pi_0$) of the marker-trait effects are real. Figure 3 shows four graphs; a graph estimating π_0 , p-values plotted against q-values, q-value cut-off against the number of significant tests and the number of significant tests against the expected number of false positives. The maximum estimated q-value (=FDR, when calling all p-values at the chosen level significant) for the stringent Bonferroni corrected p-value of 0.0007 is 0.03. For a p-value of 0.05 it is 0.28. The

largest p-value for a FDR of ≤ 0.05 is 0.002, which is less stringent than the Bonferroni corrected p-value of 0.0007. If a FDR of 5% is accepted, 59 marker-trait associations are deemed significant. Out of these about 3 are thus expected to be false positives. Table 2 shows the differences in the number of tests deemed significant when accepting FDR of 5% instead of the p-value of 0.0007. As can be seen from Figure 3, the number of expected false positives remains fairly low until about 100 significant tests. The FDR is about 10%, and thus among the 100 significant tests about 10 are expected to be false

Table 2. The traits measured in wild barley accessions, the number of marker-trait correlations at 0.05 and 0.0007 significance levels and at the FDR of 5%.

Trait	Number of marker-trait correlations		
	P<0.05	P<0.0007	FDR of 5%
Growth related			
Total dry mass (TDM)	6	1	1
Leaf area ratio (LAR)	2	0	0
Leaf mass fraction (LMF)	10	1	2
Relative growth rate (RGR)	8	1	2
Root mass fraction (RMF)	10	1	2
Specific leaf area (SLA)	1	0	0
Stem mass fraction (SMF)	10	0	0
C and N economy related			
Carbon content (C)	7	0	0
Chlorophyll	10	0	1
Mineral content	10	1	1
Nitrogen content (N)	5	1	1
NO ₃	6	0	1
Organic acids	6	0	0
Organic nitrogen/leaf area (LNC)	8	0	0
C/N ratio (CN)	6	1	1
Osmotic potential	1	0	0
Photosynthesis/leaf area (PS _A)	12	0	1
Photosynthesis/leaf mass (PS _M)	8	1	2
%Respiration	2	0	0
Root respiration (RR)	8	0	0
Shoot respiration (SR)	1	0	0
Water use efficiency (WUE)	6	0	0
Morphological			
Height	12	0	3
Leaf Angle	11	1	1
Leaf area	7	0	1
Leaf mass density (LMD)	10	0	5
Leaf thickness	10	2	4
Leaf width	17	6	8
Number of tillers	14	3	6
Seed mass	16	5	7
Water content leaf (WCleaf)	13	4	5
Water content root (WCroot)	3	1	1
Water content stem (WCstem)	11	2	3
Total	267	32	59

positives. This could also be taken as an alternative cut-off point instead of a fixed p-value.

Discussion

Variation in RGR and its underlying components have been studied extensively, but to what extent these traits are genetically linked and/or controlled by common factors is still largely unknown. A QTL analysis was

carried out to determine the genomic regions that control these traits and to assess the overlap between QTL for different traits (Poorter *et al.* 2004). In the present study we compared the QTL found for growth-related traits with the marker-trait associations in 81 accessions of *H. spontaneum*. A number of linkage groups in the 'Ashqelon x Mehola' map could be assigned to a specific chromosome due to markers identical with those found for *H. vulgare*. However,

in some cases there was only one common marker, which complicates the comparison between the two approaches. Fortunately this affected the comparisons of only two QTL on chromosome 2.

Marker-trait associations for growth-analysis traits

A total of eight markers were associated with RGR. The marker that showed the strongest correlation with RGR explained 15% of the variation and was mapped on chromosome 2. One of the other markers that correlated with RGR was located in the middle of a RGR QTL on chromosome 1 and explained 11 % of the total variation.

SLA is the most important trait explaining differences in RGR in many studies (see for a review Poorter & Van de Werf, 1998) and it has the highest correlation with RGR (Poorter *et al.* 2004). However, there was almost no difference in SLA in the 81 accessions (Van Rijn *et al.* 2000), and no significant difference between the 21 populations. In contrast with these results, the F₃ population showed substantial differences in SLA (ranging from 33 to 45 m² kg⁻¹). Only one marker (on chromosome 7) was correlated with SLA among the accessions. The only QTL for SLA was detected at the same location as the marker-SLA association on chromosome 7. Even though there was a difference between the SLA measurements on the population and the cross level, both of the resulting associations point at the same location.

Marker-LMF associations supported two out of the four LMF QTL. LMF is usually not the most important factor explaining differences in RGR, but is sometimes positively correlated with RGR (Ingestad, 1981; Poorter & Remkes 1990), sometimes negatively (Hunt *et al.* 1987; Shipley & Peters 1990; Van Rijn 2001).

Marker-trait associations for C and N economy-related traits

There were 12 markers associated with PS_a and eight associated with PS_m. PS_a is positively correlated ($p < 0.001$) with G_s and ULR, and negatively ($p < 0.001$) with LAR (Van Rijn 2001). QTL for these traits coincide on chromosome 4, where G_s, ULR and PS_a show a positive effect of the Ashkelon allele and LAR shows a positive effect of the Mehola allele. Three markers correlated with PS_a were mapped on chromosome 4, two of these supported the QTL and one was just outside the QTL interval. These four markers together explained 32% of the total variation. Note that photosynthesis was determined differently in Poorter *et al.* (2004), where it was measured on a single leaf. In this study, photosynthesis was determined on the whole plant. The fact that both measurements point to the same direction makes these observations relatively robust. Another interesting detail is that a QTL for chlorophyll content was also found on chromosome 4 in barley (This *et al.* 2000). Poorter *et al.* (2004) did not measure chlorophyll content, but in the population study it

was measured and the marker that showed the best correlation with chlorophyll content ($r = 0.36$) was also located on chromosome 4. These results, together with the mapping of a Rubisco activase gene on chromosome 4 in *H. spontaneum* (Becker & Heun 1995) show that this location on chromosome 4 might be very important for area-based photosynthetic traits.

Marker-trait associations for morphological traits

Strong marker-trait associations support the QTL for leaf width, seed mass and leaf angle on chromosome 2. There are three other morphological QTL on this chromosome, height, leaf length and root length, as well as a QTL for RGR. Leaf and root lengths were not measured in the population study. Several markers are strongly associated with seed mass, leaf thickness and leaf width on chromosomes 1, 2 and 4. QTL for seed mass and leaf width coincide on chromosome 2 and QTL for leaf width and leaf length coincide on chromosome 4. They are also correlated with each other (Van Rijn 2001). These results suggest that these morphological traits are at least genetically linked, but maybe also controlled by a common factor. Because dwarfing genes have been mapped in barley on chromosome 2 (Börner *et al.* 1999) and on chromosome 4 (Ivandić *et al.* 1999), a GA controlled gene might be a likely candidate. However, these genes

were mapped in cultivated barley and this does not necessarily mean that those alleles are also present in *H. spontaneum*.

Conclusions

Marker-trait associations in 21 wild barley populations support several QTLs mapped by Poorter *et al.* (2004). Marker trait associations supported two out of four RGR QTL. However, no association with SLA was found at these locations. The QTL for SLA and its supporting marker-trait association is found on chromosome 7 with a negative association with LMF. Markers associated with PS_a and chlorophyll content were mapped on chromosome 4, where also QTL for PS_a and stomatal conductance were found. Another cluster of photosynthesis traits PS_m and PS_a QTL and marker-trait associations were found on chromosome 5. Many markers are associated with leaf thickness, leaf width and seed mass, suggesting that these traits are tightly linked.

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Chapter 5

The island lies like a leaf upon the sea.
Green island like a leaf new-fallen from the tree.
Green turns to gold,
as morning breeze gently shakes the barley,
bending the yellow corn.
Green turns to gold,
there's purple shadows on the distant mountains,
sun in the yellow corn.

Christy Moore – Green Island



Quantitative trait loci for seed dormancy in wild barley (*Hordeum spontaneum*)

Tytti K. Vanhala & Piet Stam

Abstract

*A quantitative trait locus analysis was carried out to unravel the genetic basis of dormancy in wild barley (*Hordeum spontaneum*) from Israel. Two accessions, 'Ashkelon' and 'Mehola', from divergent environments were crossed to produce a mapping population. A linkage map was produced from the F_2 population, and F_4 seeds were used for germination experiments. Five quantitative trait loci (QTL) were detected for dormancy across the different germination experiments. These QTL were found on chromosomes 1, 2, 5, 6 and 7. The variation explained by each QTL varied between 8% and 25%. Ashkelon alleles increased the germination except for the QTL on chromosome 5.*

Introduction

Seed dormancy is an important trait not only for the agricultural industry but also for seedling survival in wild species. It has been well studied in several crop species, such as barley, wheat, oat and rice (for a review see Foley and Fennimore 1998). A seed is dormant when it fails to germinate even though it is mature and conditions for germination are optimal. Dormancy can be due to the embryo or the seed coat (Bewley and Black 1994). Embryo dormancy is lost first and therefore it is

the seed coat dormancy that ultimately determines when the seed germinates (Rodriguez *et al.* 2001). Although nondormant seeds are desirable in the malting industry, these might sprout in the ear in wet conditions before harvesting and therefore reduce yield and seed quality. Highly dormant seeds, on the other hand, create storage problems during their after-ripening period as well as being a weed problem.

The level of dormancy does not depend only on the genetic make-up of the seed but also on the environmental growing conditions of the maternal plant

during the seed maturation process, although this seems to depend also on the cultivar (Schuurink *et al.* 1992). Germination success is also dependent on the environmental conditions during germination process. Especially temperature (Fennimore *et al.* 1998) and water saturation level (Thomas *et al.* 1996) play a crucial role.

Several quantitative trait loci (QTL) have been detected in cultivated barley for seed dormancy (Ullrich *et al.* 1992; Oberthur *et al.* 1995; Han *et al.* 1996; Thomas *et al.* 1996). A major QTL (SD1) has been found on chromosome 7, explaining 36% of the variation in dormancy (Ullrich *et al.* 1992). In the same cross three minor QTL were found on chromosomes 1, 4 and 7 (Ullrich *et al.* 1992).

Dormancy is much stronger in wild than in cultivated barley (Ogawara and Hayashi, 1964; Gutterman *et al.* 1996). The removal of the glumellae and the husk enhance the germination greatly (Gutterman *et al.* 1996; Wang 1997). This seems to be largely due to the increased abscisic acid (ABA) diffusion from the seed (Wang 1997). The duration of after ripening in wild barley depends on the population; the more extreme the habitat the longer it takes for the dormancy to break (Gutterman and Nevo, 1994). This has an important effect on seedling survival in drier areas where germination after unusual rain during the dry season would be fatal.

The aim of this study was to detect QTL for seed dormancy in wild barley and to compare the results to the QTL found in cultivated barley.

Material and Methods

Plant material

A single pair cross between two accessions of *Hordeum spontaneum* from Israel was made. The paternal plant was from the site Ashkelon, on the Mediterranean coast, and mother plant from Mehola, in the Jordan valley (Chapter 2). We refer to the accessions as Ashkelon and Mehola, after the site of origin. Ashkelon has a moderate level of dormancy whereas Mehola is highly dormant, requiring a long after ripening period. F₁ and subsequently F₂ were left to self-fertilise. Ten F₃ seeds per one F₂ plant were pooled and grown together in a large (25 cm diameter) pot in order to produce F₄ seeds. In the remainder of this chapter the bulked F₄ seeds deriving from a single F₂ plant are referred to as a line. The plants were grown in a non-heated greenhouse, sown autumn 1999 at the Ecological Institute, Heteren, The Netherlands.

The F₄ seeds were collected randomly from all the ten F₃ plants. Because wild barley has a brittle rachis, complete synchronisation of the seed ripeness was not possible. Instead, seeds were collected when they were getting loose from the ear. Already

dropped seeds were excluded and seeds that were not loose were left to ripen further. The loose seeds were collected approximately every 3 days during a four-week period in June 2000 from 102 lines. The seeds were counted per line, let to air dry further at +15°C, 30% humidity for approximately 3 days, then sealed in plastic bags and stored at -20°C until the germination experiments could be started.

Germination experiments

Six germination experiments were conducted with 14-day intervals. Forty F₄ seeds per line were used for every germination experiment. The germination experiments were conducted using intact dispersal units, *i.e.* the husks were not removed. For the first germination experiment, seeds were let to thaw at room temperature over night before placing them on petri dishes between two water-saturated blotting papers. The remainder of the seeds were put in paper envelopes and after-ripened in a dry oven at +40°C. Batches of seeds were taken from these envelopes every 14 days to germinate them. The germination was done at +10°C for five days then at +20°C for 10 days, dark. Germinating seeds were counted and removed from the petri dishes after five, seven and 10 days at +20°C. The seeds were counted and removed from the petri dishes this way in order to avoid overcrowding. The

total amount of germinated seeds per line was used in the QTL analyses.

AFLP map

A linkage map using dominant and co-dominant AFLP-markers and a few microsatellite markers was constructed from the F₂. The map covered approximately 45% of the wild barley genome due to unexpectedly high heterozygosity of the Ashkelon parent (Chapter 2). Two hundred and two (202) markers were mapped on 11 linkage groups. This full map was used as a basis in the QTL analysis.

Statistical analyses

Germination percentages were calculated from the germination data. Distributions of the germination percentages were non-normal and therefore arcsin square root transformations were applied (Figure 1). The QTL analysis was performed using the transformed data.

QTL analysis was conducted using MapQTL 4.0 (van Ooijen & Maliepaard 1996). Interval mapping (IM) was used at first, multiple QTL mapping (MQM) was performed after that with inclusion of cofactors. Cofactors were selected to be markers with LOD score above 2. When the LOD values stabilised in MQM, restricted MQM (RMQM) was performed with the cofactors and these values were taken as the result of the mapping procedure. A LOD threshold value of 2.7 for a

significant QTL was used based on Van Ooijen *et al.* (1999). The estimated dominance effects obtained from MapQTL were multiplied by two to correct for the different generations in which marker data and phenotypic data were collected, *i.e.* F₂ and F₄, respectively (cf. Verhoeven 2003).

Results and discussion

Germination percentages were calculated for each germination experiment (from day 0 to day 70). Five lines started to germinate (2.5–5%) on day 0. For a different set of five lines the onset of germination was on day 14 (2.5-7.5%). On day 28, 85% of the lines had started to germinate (2.5-75%). Only two lines remained completely dormant on day 42 and on day 56 all the lines were germinating (24-100%). Complete breaking of dormancy with 100% of germination was first observed for three lines on day 42. After 70 days of after-ripening, 42% of the lines reached germination of 90% or over.

The average germination percentages are shown in Figure 2. They are divided into three groups, *i.e.* (a) the total sample, (b) lines continuously increasing in germination and (c) lines decreasing in germination between days 42 and 56. The reasons for this division are explained below.

The breaking of dormancy was more or less continuously increasing in 67% of the lines (group b; Figure 2). Within these lines, the germination percentage increased or remained the same with prolonged after-ripening. However, 33% of the lines experienced a reduction in germination on day 56 in comparison to the germination percentage of day 42 (group c; Figure 2). The most extreme line germinated only by 7.5% on day 56 after germination of 65% on day 42. On day 70, germination increased to the same level as it was on day 42 or exceeded that level substantially. Surprisingly, four lines did not seem to 'recover' but germinated less than on day 42. One of these four lines germinated even less than on day 56. Ogawara and Hayashi (1964) observed similar reduction in germination in their experiments between days 87 and 101. However, these authors did not observe such a decrease in germination in dehusked or cut seeds. This would point towards the involvement of the husk in the decrease of germination. Perhaps there is a critical after-ripening temperature and time effect on germination in nature related to survival. The seeds used in this study were intact caryopses and perhaps the hydration level of the seed coat together with the plant hormone metabolism inhibited germination of the 'decreasing' lines. This inhibitory effect, however, is mostly lost after further 14 days of after-ripening.

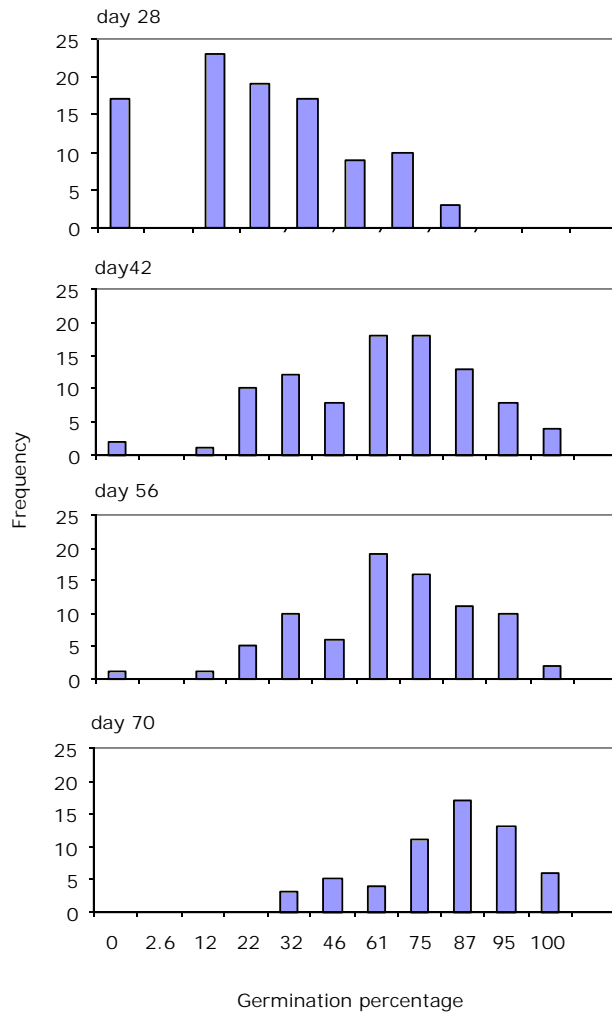


Figure 1. Frequency histograms of the transformed germination percentages (arcsin square root) of the F4 seeds in the four germination experiments used in the QTL analysis. On the x-axis the germination percentages for the different classes have been back transformed.

Another possibility is that by chance part of the seeds sampled on that day were not viable and therefore the germination percentage seemed to be decreasing. However, no selection of seeds was conducted when taking them out of the paper bags and therefore it is highly unlikely that the proportion of

non-viable seeds varies greatly among successive random samples taken from the same line, in a pattern that exactly corresponds to the observed decrease in germination. The viability of non-germinated seeds was not tested. This was assumed not to vary between the lines.

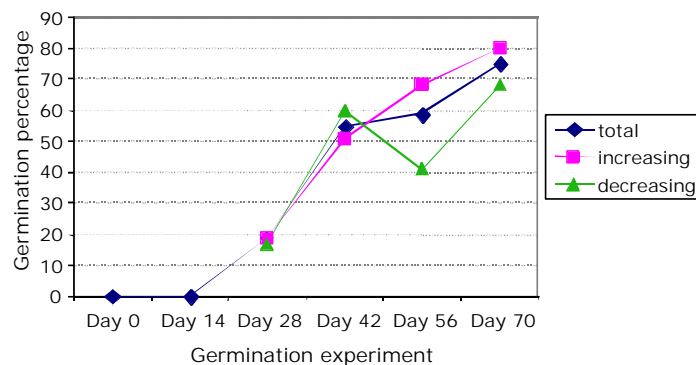


Figure 2. Average germination percentages for each germination experiment in total and divided in increasing in germination and decreasing in germination lines.

These explanations for the reduction of germination percentage on day 56 remain speculative and further research is needed to establish the reason behind these results in intact wild barley seeds. It would not be surprising, if this trend is not observed in all accessions or populations, as in the F_4 population of this study only one third of the lines showed this reduction.

Seeds from parental accessions harvested at the same time as the F_4 seeds were germinated alongside the mapping lines. Ashkelon seeds first broke dormancy on day 28 (48% germination) whereas Mehola seeds had to be after-ripened until day 70 before any germination (15%) was observed. No F_4 line was more dormant than Mehola.

There were marked differences in the rate of the release of dormancy between lines. One line lost its dormancy fast after 28 days after-ripening, gaining 100% by day 42.

Another line stayed dormant having low but increasing germination percentages reaching 24% by day 70. Yet another example is a line which did not germinate until day 56 reaching 95% germination on day 70.

QTL mapping

QTL mapping was conducted on the transformed data for days 28, 42, 56 and 70. In total five QTL with LOD scores above 2.7 were detected across the experiments. The QTL were found on chromosomes 1, 2, 5, 6 and 7. Table 1 summarises the QTL results.

QTL mapping was performed at first using the full map data with 202 markers across 11 linkage groups (Chapter 2). Close inspection of the marker data revealed that 51 markers had a very low information content (less than 10 F_2 plants with known genotype). These markers were removed from the data set and thus all subsequent QTL analyses were

performed using the remaining 151 markers.

A QTL was detected on chromosome 1 for day 70. The significance of this QTL is fairly high (LOD 3.95) but it is based on information only from one marker class and thus is rather suspect. To find out whether there was real lack of other allele classes or whether more information could have been obtained on this occasion by using the full sample size of 233 F_2 derived F_4 lines, we checked the information content of the surrounding markers in the full sample. The results show that no more information could be gathered in this way; the nearest balanced marker remains 10 cM away from the highest LOD peak. This distortion in the marker data could be due to segregation distortion. But, as noted in Chapter 2, due to the heterozygosity of especially the Ashkelon parent many homozygous markers per F_1 'family' were removed from the data. Therefore little can be said about the segregation distortion in this mapping population.

Ullrich *et al.* (1992) detected a QTL for seed dormancy on chromosome 1 (SD3) associated with the marker *Amy2* (position 85 cM on barley consensus map; Qi *et al.* 1996). Unfortunately only one of our AFLP markers on this chromosome is shared with the cultivated barley map, and therefore we cannot determine the orientation of the chromosome. Thomas

et al. (1996) found two QTL on chromosome 1. One of these (near marker PBI12) might also be the SD3 QTL.

On chromosome 2A, a QTL was found for days 28 and 56 (LOD 2.90 and 3.16, respectively). The highest LOD peaks per experiment were found in the middle of the chromosome, although LOD peaks over 2.7 were also detected at the top of the chromosome and thus might point towards a possible second QTL on this chromosome. The QTL explained 10% and 12% of the variation and was consistently additive. The Per1 gene is involved in dormancy release (Stacy *et al.* 1996) and is nearby a marker B15C on chromosome 2 position 66cM on the barley consensus map (Qi *et al.* 1996). The QTL found in this study on chromosome two is around this position. Ullrich *et al.* (1992) detected a QTL also near the marker B15C (ABC306, 69 cM on barley consensus map). Thomas *et al.* (1996) mapped two QTL on this chromosome (PBI21a, 26 cM and CDO64, 49 cM), but not around the Per1 gene.

On chromosome 5, a QTL was detected for day 56 (LOD 2.75). This was the only QTL of which the Mehola allele enhanced germination. Thus even though Mehola is very dormant, it still contains alleles for inducing germination. The QTL explained 12% of the variation and was overdominant.

On chromosome 6 we detected a QTL for days 28 and 42. Thomas *et al.*

Table 1. QTL for transformed germination data. Experiment, map location, LOD values, percentage of variation explained by the QTL, additive and dominance effect of the QTL, and the associated marker. The dominance effect is corrected for F₄ as described by Verhoeven (2003).

Exp.	map location		LOD	% expl	add. effect	dom. effect	nearest marker
	Chr.	cM					
day 28	2A	35	2.90	10.1	0.12	-0.08	E38M54-169
	6	14	3.46	13.3	0.10	0.20	E38M54-349
	7	12	4.57	15.4	0.15	0.04	E39M61-255
day 42	6	14	4.84	21.5	0.22	-0.02	E38M54-349
	7	12	3.39	14.6	0.17	-0.04	E39M61-255
day56	2A	31	3.16	12.6	0.13	-0.18	E33M55-436
	5	53	2.75	11.7	-0.04	-0.38	E33M55-591
	7	11	2.11	8.6	0.12	-0.06	E33M61-221
day 70	1	42	3.95	25.0	0.20	0.08	E38M58-84

(1996) detected a QTL for dormancy around this position as well. The chromosome is linked to the cultivated barley map only with one marker, so the orientation is not resolved. The QTL explained 13% and 22% of the variation for the two different days, respectively. QTL x time interaction was observed at this QTL; for day 28 the QTL was completely dominant whereas for day 42 it was additive.

On chromosome 7 a QTL was detected for days 28 and 42. A putative QTL was detected at the same position also for day 56 (LOD 2.11). The variance explained by the QTL was about 15% for both days 28 and 42, whereas the putative QTL on day 56 explained 9% of the variation. The QTL had a consistent additive effect across the experiments. High LOD peaks were

also observed on both sides of the detected QTL. These were considered to be rather suspect as markers at both ends of the chromosome are only of one marker class as was the case with the QTL on chromosome 1. Extending the population to the full set of 233 F₂ plants it appeared that no more information could be obtained for these markers. Therefore, it is not possible to determine whether there are two more QTL on this chromosome or not. To solve these problems, a new cross should be made with less heterozygous parents, so as to maximise the genome coverage.

Ullrich *et al.* (1992) reported a major seed dormancy QTL (SD1) on chromosome 7 explaining up to 36% of the variation in dormancy. The SD1 QTL is associated with the marker PSR128,

position 76 cM on barley consensus map (Qi *et al.* 1996). The approximate position of the SD1 is estimated to be at the end of the chromosome on the 'Ashkelon x Mehola' map. Thus the QTL detected in this study on the same chromosome is not the SD1 QTL. It could be that the SD1 QTL is expressed also in wild barley, but our map data may be failing to detect it.

Different QTL at different time points reflect a difference in the cumulative germination curves of the lines. A priori, another approach could have been taken, *i.e.* to fit an S-shaped curve to the observed cumulative germination data for each line and take the parameter(s) of those curves as the trait(s) to be analysed. However, because of the observed drop in germination percentage in a number of lines, this approach does not apply.

About one third of the F_4 mapping lines experienced a reduction in germination percentage on day 56 compared to day 42. The reasons for this reduction are unknown, although based on the results of Ogawara and Hayashi (1964) the seed coverings might have something to do with the phenomenon, as this reduction in germinability seems to be restricted to intact seeds.

To establish whether QTL mapping would detect any genomic areas associated with the reduction, a new trait was defined for the reduction in germination percentage (germination

percentage on day 56 – germination percentage on day 42) and QTL mapping was performed. One LOD peak of 2.32 was observed at one end of an unassigned linkage group U2 (see Chapter 2) explaining 30% of the variation. A LOD value of 2.3 in this linkage group corresponds with a p-value of $p < 0.011$ according to a permutation test. The reduction in germination at this locus is associated with homozygosity for the Mehola allele. Individuals being homozygous for the Ashkelon allele experience less of a reduction but heterozygotes have the largest increase in germination and thus the QTL was overdominant. It is remarkable to see that the reduction in germination between day 42 and 56 is apparently being controlled by a QTL that is not involved in the 'normal' breaking of dormancy.

Conclusions

The level of dormancy is much higher in wild barley than in the crosses studied in cultivated barley. The mapping population used in this study started germinating after 28 days of after-ripening, including the less dormant parent, whereas the highly dormant parent Mehola needed 70 days of after-ripening. Removing the husk would increase the germination of wild barley seeds (Gutterman and Nevo 1994), but we doubt whether this would be enough to allow germination of Mehola seeds immediately after harvest.

We have detected several QTL for breaking of seed dormancy in a wild barley cross. Because the 'Ashkelon x Mehola' map does not have enough markers linking it to the cultivated barley map, the QTL found here could not be fully compared with the QTL found earlier with confidence. However, some comparisons could be made revealing that three out of the five detected QTL probably correspond to QTL found earlier in cultivated barley crosses. This indicates that wild barley may harbour dormancy controlling genes that have not yet been exploited by barley breeders.

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Chapter 6

Now the ploughman he shall plough,
And shall whistle as he go,
Whether it be fair or blow,
For another barley mow,
O'er the furrow merrily:
Merrily, merrily, will we sing now,
After the harvest, the fruit of the plough.

The Barley Mow



Summarising discussion

Wild barley (Hordeum spontaneum) carries important genetic variation that is potentially useful for the improvement of barley cultivars. This is why it is of paramount importance to study wild barley accessions from different environments. To this end, different aspects of diversity, as well as linkage, QTL and association mapping in wild barley from Israel were studied in this thesis. A linkage map was constructed covering approximately 45% of the genome. This limited coverage was due to unexpected heterozygosity of the paternal parent. Diversity of 21 wild barley populations was estimated using environmental, phenotypic and genetic information. Environmental diversity was loosely associated with genetic diversity, whereas phenotypic diversity in general did not correlate with either one. Ecotype-specific markers were found. Some of these were mapped and correlated with phenotypic measurements as well as the environmental variables. Marker-trait associations were calculated and the resulting significant mapped associations plotted against results from a previous QTL study. This revealed many instances where the associations and QTL mapped to same genomic region. A dormancy study was performed in which five QTL were detected. Three of these might be the same as found earlier with cultivated barley crosses. These results of this thesis add to the characterisation of the diverse wild barley populations from Israel and aid in the understanding of the genetical backgrounds of different growth characteristics as well as seed dormancy.

Linkage mapping in wild barley

This thesis provides the first intraspecific linkage map for wild barley. The experimental 'Ashkelon x Mehola' cross was made as a single plant cross as the accessions within populations were found to be polymorphic. However, even more unexpectedly, the paternal

Ashkelon parent plant was found to be heterozygous for many markers and this decreased the maximum proportion of the genome that could be mapped to 55% (the estimated actual genome coverage is 45%). Wild barley is a predominantly inbreeding species, but outcrossing does happen (Brown *et al.* 1978a). The outcrossing event in the

Ashkelon parent has been very recent given the high marker heterozygosity that was observed.

Linking the wild barley map to cultivated barley maps is a very important basic requirement if the obtained results are to be used for cultivar improvement. The linkage mapping in Chapter 2 includes many shared markers, potentially linking the wild barley map to five different cultivated barley maps. Unfortunately, most of these markers remained unmapped in the end and only a handful of them could be used in assigning the linkage groups to known barley chromosomes. For further research and other intraspecific wild barley maps, it is important to consider genotyping with as many markers mapped in cultivated barley as possible.

Genotype, phenotype and environment

As discussed in Chapter 3, the different environments where wild barley populations grow, differ dramatically within Israel. This natural setting provides good opportunities of finding large genetic and phenotypic variation within populations for the use of barley cultivar improvement.

The 21 wild barley populations used in this thesis were studied genetically and phenotypically. At the genetic level, clear differences

were detected between the populations, whereas phenotypically no division into populations was observed. This result may be affected by the selected traits as for some morphological traits a population structure was observed. The environment, although being very varied across Israel, differs between groups of populations rather than between populations themselves. The populations from the more extreme environments (steppic and desert populations) were genetically more divergent than populations from the more stable environment (coastal and Mediterranean populations).

Genetic diversity did not correlate with the overall phenotypic diversity as expected from the opposing results concerning the differences between and within populations. However, if the traits were divided into physiological, chemical and morphological traits and analysed again, a weak correlation ($r=0.23$, $p<0.001$) emerged between genetic and morphological diversity. This is in agreement with the results from the population structure.

A number of markers were found to be ecotype-specific. These markers might be linked to genomic areas important in the adaptation of the populations to their habitat. A marker mapped on chromosome 5 (111.5 cM) had a lower band frequency within the desert ecotype. This marker correlated with several phenotypic traits among

the accessions (see Figure 1 in Chapter 4). QTL for stem mass density and height are also located near this marker as well as QTL for seed number (Mehola site; Verhoeven *et al.* 2003) and leaf length (Elberse 2002). Another ecotype-specific marker is located on the same chromosome, estimated to be near the marker at the position 111.5 cM (7.5 cM on the 'Ashkelon x Mehola' map). This in turn is associated with the steppic ecotype and correlated with chlorophyll content and water use efficiency. The ecotype specific marker on chromosome 6 (11 cM 'Ashkelon x Mehola' map) is associated with the Mediterranean populations and is coinciding with QTL for root and leaf mass fraction, root length (Chapter 4) and relative growth rate (Chapter 4; Elberse 2002) as well as seed mass (Verhoeven *et al.* 2004b; Elberse 2002), leaf width, number of roots and leaves (Elberse 2002). Chromosome 2 has also a Mediterranean ecotype specific marker, which is located on the same position as QTL for root respiration. Chromosome 4 has several QTL for photosynthesis related traits (Chapter 4) as well as an ecotype specific marker for steppic populations (49 cM). Other QTL found at this marker position include leaf area ratio, leaf width and leaf mass fraction (Chapter 4; Elberse 2002), specific leaf area (Elberse 2002), seed weight and number of seeds per head (Verhoeven *et al.* 2004b).

The results of the present population survey as well as the QTL mapping studies of Elberse (2002) and Verhoeven (2003) clearly indicate that a number of physiological traits are involved in the adaptation of wild barley to its environment. It should be noted however that Verhoeven *et al.* (2004b) did not observe QTL with opposite effects in contrasting environments. Thus, it seems that adaptation results from selection pressure that varies in magnitude among environments, but not from opposing selection pressures. Our results suggest that nevertheless these selective forces may result in ecotype-specific alleles at several loci.

Association and QTL mapping

Mapping phenotypic traits within wild populations increases the chances of locating new alleles for the use of barley breeding. Ideally, it would be advantageous to screen as many of the natural populations as possible for the desired traits. Creating several crosses between individual plants from different habitats is one option, although this is time consuming. Another approach is to use association mapping where the phenotypic traits measured across a number of populations are analysed for association with individual markers which are already mapped either in wild or cultivated barley crosses. Association mapping gives more information in

some aspects compared to QTL mapping, because it is not restricted to the genetic make up of the two parents that are used to make an experimental cross for linkage mapping. The results from association mapping can also be used as a criterion for selecting parental accessions for another cross with individuals from accessions carrying different alleles for the marker-trait associations of interest. This way association mapping can be effectively used to screen a large number of populations and aid the decision process for further research with these populations.

In this thesis, for several traits, QTL detected in the experimental cross (Poorter *et al.* 2004) and associations detected across populations (Chapter 4), map to the same genome regions. This co-localisation underlines that association mapping is a useful alternative to the use of segregating mapping populations, provided that the linkage disequilibrium causing association is not the result of forces other than linkage itself.

Dormancy

Dormancy remains an important trait in barley breeding. Most of the varieties have lost their dormancy completely, although some cultivars exhibit still some amount of dormancy. Ideally, the cultivars should have a level of dormancy preventing preharvest

sprouting but low enough to not to cause problems in the form of extended storage or weeds in the following crop cycle. Wild barley is known to show dormancy up to much higher levels than what is observed in cultivated barley. Therefore, studying dormancy within a wild barley cross might offer several new alleles involved in dormancy maintenance and release for the breeding of optimally dormant cultivars

However, studying dormancy in wild barley is not as straightforward as it is in cultivated barley. The main difficulty is the brittle rachis. This makes it virtually impossible to synchronise the ripeness of the seeds as one would do with cultivated barley. Another difference to cultivated barley is the husked seed. This is important to take it into account as the husk is related to the level of dormancy.

Chapter 5 records the first attempt to discover whether the dormancy QTL found in cultivated barley are also found within a wild barley cross. Five QTL were detected across different chromosomes. Three of these QTL could be the same as detected earlier in cultivated barley, thus leaving two new dormancy QTL.

It is clear that the dormancy QTL analysis has suffered from incompleteness of the linkage map. A linkage map covering the whole wild barley genome would have provided more markers in common with other mapping populations, enabling a more

thorough comparison with dormancy QTL studies in cultivated barley.

Complementary to QTL studies of dormancy in mapping populations, association mapping using mapped markers, as described in Chapter 4 for RGR-related traits could provide an interesting overview of the presence of dormancy genes in the populations across the whole of Israel.

Conclusions

Wild barley populations are diverse both genetically and phenotypically. Cultivated barley, on the other hand, has lost much of its variation through breeding. As wild and cultivated barley produce fully viable hybrids with normal chromosomal pairing, the larger variation detected in wild barley can be used to improve barley cultivars.

The wild barley populations from Israel studied here were diverse in all levels; genetic, phenotypic and environmental. The genetic variation was larger between populations than within them, whereas phenotypic variation was larger within populations than between them. The environment differed between populations, although the difference was more notable between groups of populations, designated as ecotypes.

An intraspecific molecular linkage map was constructed with the aim to locate QTL involved in growth-

related traits as well as dormancy. Although this linkage map does not cover the whole wild barley genome, a number of QTL have been located which, in principle, enables efficient introgression of favourable genome segments from wild barley into the cultivated barley genetic background.

This linkage information was complemented by a diversity study of wild barley populations from Israel, both at the DNA and the phenotypic level. Although the pattern of intra- and interpopulation diversity reflected by phenotypic and genotypic differences were not parallel, all the traits studied were associated with at least one mapped molecular marker. Many of these trait-associated markers are located in the same genomic regions as QTL underlying these traits detected in mapping populations. This concurrence illustrates the potential usefulness of association mapping for breeding purposes. Lastly five QTL for dormancy were detected. At least two of these appear to be novel QTL, not yet detected in cultivated barley.

Samenvatting



Samenvatting

In het Kort

Wilde gerst (Hordeum spontaneum) vormt een belangrijke genetische bron voor cultuurgerst, die, als gevolg van intensieve veredeling, een beperkte genenpool heeft. Het is daarom van belang om de genetica van verschillende eigenschappen van wilde gerst te bestuderen, als men deze wil gebruiken voor de verbetering van gerst. Dit proefschrift beschrijft onderzoek aan de verschillende aspecten van diversiteit, genetische koppeling, QTL- en associatie-analyse bij wilde gerst. De natuurlijke populaties die zijn bestudeerd, zijn verzameld in diverse habitats in Israël. De nakomelingen van een kruising tussen twee accessies uit contrasterende habitats zijn gebruikt om een koppelingskaart van moleculaire merkers te maken. Wegens een onverwacht hoge graad van heterozygotie van één der kruisingsouders bedekte de kaart 45% van het gerstgenoom. Deze moleculaire merker kaart vormt de basis voor de vervolgstudies die in dit proefschrift beschreven zijn. De genetische diversiteit binnen en tussen 21 populaties is bestudeerd. Deze is vervolgens gerelateerd met diversiteit gebaseerd op milieukarakteristieken en 33 eerder gemeten groei-eigenschappen. De genetische diversiteit was licht gecorreleerd met de milieu diversiteit. In het algemeen was er geen significante correlatie tussen enerzijds de genetische en milieudiversiteit en anderzijds de kenmerkdiversiteit. Enkele merkers waren specifiek voor een bepaald milieutype. Sommigen van deze merkers waren eerder in kaart gebracht en behalve met milieueigenschappen waren zij ook significant gecorreleerd met groei-eigenschappen. Om na te gaan of de waargenomen genetische differentiatie wellicht parallel loopt aan de fenotypische differentiatie, zijn correlaties geschat tussen de gekarteerde merkers en individuele eigenschappen, gemeten in dezelfde 21 populaties. De significante associaties zijn vervolgens vergeleken met eerder gevonden QTL. Veel van deze associaties vallen samen met dezelfde genoomregio's als de QTL, hetgeen illustreert hoe deze twee verschillende analyses elkaar complementeren. In het laatste deel van dit proefschrift, is de moleculaire merker kaart gebruikt om QTL voor kiemrust bij wilde gerst te vinden. Er werden vijf QTL gevonden, waarvan drie waarschijnlijk eerder zijn beschreven bij cultuurgerst. Voor verder onderzoek is het raadzaam om een nieuwe kruising op te zetten met als doel een kaart te verkrijgen die het gerstgenoom beter bedekt. De resultaten van dit proefschrift dragen bij aan de beschrijving van de genetische aspecten van diverse wilde gerst populaties uit Israël, en vergroten het inzicht in hun potentie voor verbetering van cultuurgerst.

Wilde gerst

Wilde gerst is de voorouder van cultuurgerst. Hij behoort tot de Poaceae- familie en binnen deze familie tot de Triticeae. De Triticeae vertegenwoordigen een plantengroep van de gematigde klimaatzone, voornamelijk geconcentreerd in Centraal en Zuidoostelijk Azië, hoewel de soorten uit dit taxon over de hele wereld voorkomen. Binnen de Triticeae treffen we veel economisch belangrijke granen en ruwvoedergewassen aan, naast ongeveer 350 wilde soorten. De wilde soorten zijn van groot belang als potentiële genetische bronnen voor commerciële veredeling.

Het genus *Hordeum* omvat 30 soorten (Von Bothmer 1992), zowel één- als meerjarigen. Afhankelijk van het locale klimaat is wilde gerst van het winter- of zomertype (al dan niet vernalisatiebehoefstig). Het is een diploïde ($2n=14$) en meestal zelfbevruchtende plant, hoewel sommige varianten een zekere mate van kruisbevruchting vertonen. Het gemiddelde kruisbevruchtingspercentage is 1.6%, variërend van 0 tot 9.6%, afhankelijk van de standplaats. In de gematigdvochtige regio's is het percentage kruisbevruchting doorgaans hoger dan in de droge regio's van het verspreidingsgebied (Brown et al. 1978a).

De wilde éénjarige *Hordeum* soorten komen voor in open habitats met

relatief weinig concurrentie van andere soorten (Von Bothmer 1992). Wilde gerst is verspreid over zeer diverse klimaatgebieden en bodemtypes in het oostelijk Middellandse zee gebied en in zuidoost Azië. Wilde gerst wordt vooral aangetroffen in de zogenaamde 'Near East Fertile Crescent', een gebied in het nabije Oosten dat zich, min of meer sikkelvormig, uitstrekt van het oostelijk Middellandse zeegebied tot in Irak en waar de eerste vormen van landbouw zijn ontstaan (Zohary 1969). In het algemeen is wilde gerst niet bestand tegen extreme koude en wordt dan ook zelden aangetroffen boven 1500 meter hoogte. De droogteresistentie daarentegen is beter dan die van Emmer (wilde tarwe) en de wortels dringen diep in de bodem van de warme steppes en woestijnen (Zohary & Hopf 1988).

Wilde gerst en twee-rijige cultuurgerst hebben een vergelijkbare morfologie. De opvallendste verschillen bij wilde gerst zijn de omhulde zaden en de broze aarspil, waardoor de rijpe zaden op de grond vallen. Zes-rijige gerst is ontstaan gedurende de domesticatie van gerst; het kenmerk wordt veroorzaakt door een hoofdgen op chromosoom 2 (Komatsuda et al. 1999; Tanno et al. 2002). Wilde gerst is de enige wilde *Hordeum* soort die volledig vruchtbare hybriden oplevert na kruising met cultuurgerst (met normale chromosoomparing en segregatie in de meiose). Deze hybriden komen ook van

nature voor wanneer wilde en cultuurgerst in dezelfde omgeving voorkomen (Asfaw & Von Bothmer 1990).

De collectie van wilde gerst uit Israël is bijeengebracht tussen 1974 en 1976 (Nevo et al. 1979). Gedurende deze periode zijn duizenden individuele planten (accessies) verzameld vanuit geheel Israël. Op uiteenlopende plaatsen zoals wegbermen, berghellingen, akkers en woestijnen zijn monsters genomen. Lengtegraad, breedtegraad, hoogte, alsmede neerslag-, vochtigheids- en temperatuurgegevens werden verkregen van nabijgelegen weerstations en zijn toegevoegd aan de collectiegegevens. Deze collectie wordt beheerd door professor Eviator Nevo en zijn medewerkers op het Institute of Evolution in Haifa, Israël. In de daaropvolgende jaren zijn extra monsters, afkomstig uit Tabigha (Nevo et al. 1986) en de zogenaamde "Evolution Canyon" (Nevo et al. 1995) toegevoegd aan de verzameling. Accessies van deze verzameling in Haifa zijn over de hele wereld verspreid voor onderzoek. Omdat er zoveel accessies beschikbaar zijn is er echter maar weinig overlap tussen de verschillende studies die gebruik maken van deze collectie.

Wilde gerst populaties zijn zowel genetisch als fenotypisch zeer gevarieerd. Cultuurgerst, daarentegen heeft veel van zijn variatie verloren als

gevolg van selectie. Omdat wilde en cultuurgerst probleemloos gekruist kunnen worden, kan de grotere variatie in wilde gerst worden benut om cultuurgerst te verbeteren. Daarom is het van belang om wilde gerst variëteiten van verschillende locaties grondig te bestuderen. Via het bestuderen van de diversiteit, het maken van een genetische merker kaart, alsmede QTL- en associatiestudies levert dit proefschrift een belangrijke bijdrage aan de kennis over wilde gerst in Israël.

Moleculaire merker kaart van wilde gerst

Een merkerkaart van wilde gerst is gemaakt met de F₂ generatie uit de kruising tussen de accessies 'Ashkelon' en 'Mehola'. Daarbij zijn dertien microsatelliet merkers en 22 AFLP primer combinaties gebruikt, resulterend in 384 informatieve merkers waarvan er uiteindelijk 202 geplaatst konden worden op 11 koppelingsgroepen. De kaart omspant 445 centiMorgan (cM) met een gemiddelde afstand van 2.2 cM tussen de merkers. Om de koppelingsgroepen toe te wijzen aan beschreven gerstchromosomen is gebruik gemaakt van 33 AFLP merkers die eerder in kaart waren gebracht in vier verschillende kruisingen van cultuurgerst. Ook van de microsatellieten was bekend tot welk gerstchromosoom zij behoren. Omdat één van de kruisingsouders

heterozygoot bleek te zijn voor een groot aantal merkers, beslaat de huidige kaart ongeveer 45% van het wilde gerst genoom. Deze kaart is de eerste intraspecifieke merkerkaart van wilde gerst.

Genetische, fenotypische en milieu variatie in wilde gerst

De genetische en fenotypische variatie is geschat voor 72 Israëlische accessies afkomstig van 21 populaties. Deze populaties kunnen worden opgedeeld in vier milieutypes. De lokale milieuv variabelen zijn gebruikt om de milieudiversiteit te schatten. Genetische, fenotypische en milieuafstanden zijn geschat en vervolgens gebruikt voor de constructie van UPGMA dendrogrammen. Tussen populaties bleek de genetische variatie, zoals weerspiegeld door moleculaire merkers, groter te zijn dan binnen populaties, terwijl de fenotypische variatie binnen populaties groter was dan die tussen populaties. Er was geen significante correlatie tussen genetische en fenotypische afstanden noch tussen fenotypische en milieuafstanden. Er was enige mate van correlatie tussen genetische en milieuafstanden. Drieëntwintig AFLP-merkers waren specifiek voor een bepaald milieutype. Voor vijf van deze merkers was ook de chromosoomlocatie bekend. Van deze vijf waren vier merkers significant gecorreleerd met fenotypische kenmerken en milieuv variabelen.

Tesamen met de resultaten verkregen in een andere studie werpt deze analyse licht op het mechanisme dat ten grondslag ligt aan genotypische en fenotypische differentiatie tussen de wilde gerst populaties.

Associatie en QTL analyse

Quantitative trait loci (QTL) voor uiteenlopende fysiologische eigenschappen, die gevonden zijn in de kruising van twee wilde gerst lijnen zijn vergeleken met merker-kenmerk associaties die geschat zijn aan 81 wilde gerst accessies. Aan deze accessies zijn 33 kenmerken gemeten met betrekking tot groei, stikstof- en koolstof-efficiëntie alsmede plantmorfologie. De accessies zijn getypeerd voor 68 AFLP merkers, die eerder in kaart waren gebracht in de genoemde wilde gerst kruising dan wel in een kruising tussen gerstrassen. Van deze 68 merkers, vertoonden 60 een significante correlatie met één of meerdere kenmerken. Merker-kenmerk associaties bevestigden een aantal van de eerder beschreven QTL resultaten. Voor relatieve groeisnelheid werden twee van de vier QTL bevestigd door merker-kenmerk associaties. Vier merkers met een effect op fotosynthese per bladeenheid lagen op chromosoom 4, waar ook een QTL voor fotosynthese en stomata-geleiding gevonden was. Eén van deze merkers heeft ook een effect op chlorofyl hoeveelheid wat suggereert dat dit gebied op chromosoom 4 erg

belangrijk is voor kenmerken die zijn gerelateerd aan fotosynthese. Er was één QTL gevonden (op chromosoom 7) voor specifiek bladoppervlak. Dit QTL werd ondersteund door de enige merkerassociatie voor dit kenmerk. Merkers op chromosoom 1, 2 en 4 waren sterk gecorreleerd met bladdikte, bladbreedte en zaadgewicht, wat suggereert dat deze kenmerken genetisch gecorreleerd zijn.

QTL voor kiemrust

Om de genetische basis van kiemrust bij wilde gerst te ontrafelen is een QTL analyse uitgevoerd voor deze eigenschap. Voor de kiemprouven zijn

zaden van F₄-lijnen, afkomstig uit de kruising 'Ashkelon' x 'Mehola', gebruikt en diende de merkerkaart gebaseerd op de F₂ van deze kruising om QTL te localiseren. In totaal zijn vijf QTL gevonden voor de verschillende tijdstippen van het kiemexperiment. Deze QTL zijn gevonden op chromosoom 1, 2, 5, 6 en 7. Deze QTL verklaren tussen 8 en 25 % van de variatie in kiempercentage. Voor alle QTL geeft het 'Ashkelon'-allel een hoger kiempercentage, met uitzondering van het QTL op chromosoom 5. Een aantal QTL zijn uniek voor deze studie terwijl anderen mogelijk eerder gevonden QTL bevestigen.

Tiivistelmä



Tiivistelmä

Villiohra on tärkeä geeniluovuttaja viljellylle ohralle, jonka perinnöllinen tausta on kaventunut huomattavasti jalostuksen myötä. Jotta villiohraa voitaisiin käyttää ohralajikkeiden ominaisuuksien parantamiseksi, on tärkeää tutkia villiohnan erilaisten ominaisuuksien perinnöllisyyttä. Tämä väitöskirja käsittelee villiohnan muutelevuuden eri tasoja, kytkentäkartan tekoa sekä QTL- ja assosiaatiokartoitusta. Tutkitut luonnonpopulaatiot kerättiin 70-luvulla Israelista, joka on hyvin muunteleva ympäristöltään. Kaksi kasvia eri ympäristöistä risteytettiin ja molekyylärisiin merkialueisiin perustuva kytkentäkartta muodostettiin. Kartta käsitti 45% ohran genomista toisen vanhemman odottamattoman suuren heterotsygotian vuoksi. Tätä karttaa käytettiin kaikissa seuraavissa tämän väitöskirjan tutkimuksissa. Perinnöllinen muuntelu arvioitiin 21 luonnonpopulaation sisällä ja välillä. Tätä muuntelua verrattiin samojen populaatioiden fenotyypiseen ja ympäristölliseen muunteluun. Perinnöllinen muuntelu korreloi väljästi ympäristön muuntelun kanssa. Yleisesti ottaen fenotyypinen muuntelu ei korreloinut perinnöllisen eikä ympäristöllisen muuntelun kanssa. Eri ympäristöille ominaisia molekyylärisiä merkialueita löydettiin. Muutama näistä oli kartoitettu kytkentäkartalle ja ne korreloivat merkittävästi ei vain ympäristömuuttujien vaan myös fenotyypisten ominaisuuksien kanssa. Korrelaatiot kartoitettujen merkialueiden ja fenotyypisten ominaisuuksien välillä laskettiin käyttäen samoja luonnonpopulaatioita. Tilastollisesti merkittäviä merkialue-fenotyypinen ominaisuus - korrelaatioita verrattiin aiemmin kartoitettuihin QTL alueisiin. Monet näistä assosiaatioista kartoituivat samalle alueelle kuin niitä vastaavat QTL-alueet. Lopuksi kytkentäkarttaa käytettiin QTL alueiden löytämiseksi villiohnan siemenen itämislevolle. Viisi QTL aluetta löydettiin, joista kolme saattaa olla samoja aiemmin ohralla löydettyjen QTL alueiden kanssa. Tulevaisuudessa olisi hyödyllistä tehdä uusi risteytys suuremman kytkentäkartan muodostamiseksi. Tämän väitöskirjan tulokset lisäävät israelilaisten villiohrapopulaatioiden tuntemusta sekä antavat enemmän mahdollisuuksia villiohnan käyttämiseen ohralajikkeiden parantamisessa.

Villiohra

Hordeum spontaneum (villiohra) on viljellyn ohran esi-isä. Se kuuluu Poaceae –heinäkasvien sukuun ja sen sisällä Triticeae –heimoon. Triticeae on

lauhkean vyöhykkeen kasviryhmä, joka on suurimmaksi osaksi keskittynyt Keski- ja Kaakkois-Aasian alueelle, vaikka siihen kuuluvat lajit ovat levittäytyneet ympäri maailman.

Triticeae sisältää monta ekonomisesti tärkeää viljeltyä vilja- ja rehulajia sekä myös noin 350 villiä lajia. Nämä villit lajit ovat erittäin mielenkiintoisia potentiaalisina geeniluovuttajina kaupallisessa kasvinjalostuksessa.

Hordeum -suku käsittää 30 lajia (Bothmer 1992). Nämä ovat sekä yksittäisiä monivuotisia lajeja. Villiohra voi olla joko talvella tai kesällä kasvava yksivuotinen riippuen paikallisesta ilmastosta. Se on diploidi ($2n=14$) ja suurimmaksi osaksi itsesiittoinen, vaikkakin joillakin populaatioilla on korkeampi ristisiittoisuusaste. Keskimäärin tämä on 1.6%, muunnellen 0% ja 9.6% välillä, riippuen ympäristöstä; ristisiittoisuus on yleisempää alueilla, joiden maa on rehevempää verrattuna alueisiin, joiden maa on köyhää (Brown *et al.* 1978a).

Villit yksivuotiset *Hordeum* -lajit kasvavat avoimilla alueilla, joilla muiden lajien asettama kilpailu kasvualasta on vähäistä (Bothmer 1992). Villiohran alkuperäinen levinnäisyysalue ulottuu itäisen Välimeren alueelta aina Kaakkois-Aasiaan. Se kasvaa kaikenlaisissa ilmastoissa ja maaperissä. Se on erittäin yleinen Lähi-Idän Hedelmällisen Puolikuun alueella (Zohary 1969). Yleisesti ottaen villiohra on herkkä erittäin kylmille lämpötiloille, ja sitä löydetään vain harvoin yli 1500 m korkeudesta. Villiohra on kuitenkin kuivuudenkestävämpi kuin esimerkiksi villivehänä ja kasvaa myös lämpimillä

aroilla ja aavikoilla (Zohary & Hopf 1988).

Villiohra ja viljelty kaksirivinen ohra ovat aikalailla samanlaisia ulkomuodoltaan. Suurimmat erot niiden välillä ovat se, että villiohran siemenet tippuvat kypsyttyään ja se, että villiohran siemenen kuori ei irtoa siemenestä. Kuusirivinen ohra on kehittynyt domestikaation aikana, ja sitä kontrolloi geeni kromosomissa 2 (Komatsuda *et al.* 1999; Tanno *et al.* 2002). Villiohra on ainoa villi *Hordeum* -laji, joka voi risteytyä viljellyn ohran kanssa ja muodostaa täysin elinvoimaisia ja fertiilejä jälkeläisiä. Näitä villiohran ja viljellyn ohran risteymiä muodostuu myös luonnossa, jos nämä kaksi lajia kasvavat samassa paikassa (Asfaw & Bothmer 1990).

Israelilainen villiohra-kokoelma kerättiin vuosina 1974-1976 (Nevo *et al.* 1979). Tuona aikana yli tuhat eri linjaa (linjaksi kutsun tässä yhdestä kasvusta kerättyjä siemeniä) kerättiin koko Israelin alueelta. Keräyskohteina olivat monet erilaiset habitaatit, kuten tienposket, vuortenkupeet, niityt ja aavikot. Paikkakoordinaatit, korkeusaste ja erilaiset sade- ja kosteustiedot sekä lämpötilat otettiin ylös läheisiltä sääasemilta. Tätä kokoelmaa pidetään yhä yllä Evoluutioinstituutissa Haifassa. Pääkokoelmaan on sen keräysvuosien jälkeen liitetty enemmän populaatioita Tabighan alueelta Kinneret-järven rannalta (Nevo *et al.* 1986) ja Evoluutio Kanjonista Haifan lähistöltä (Nevo *et al.*

1995). Näitä villiohropopulaatioita on jaettu ympäri maailman, mutta kokoelman suuruuden takia useimmat tutkimusryhmät tutkivat eri linjoja populaatioiden sisällä, ja koska populaatioiden sisällä on muuntelua, tutkimuksia ei voi helposti vertailla keskenään.

DNA, elämän perusta

Deoksiribonukleiinihappo, eli DNA, on kaiken elämän perusta (lukuunottamatta RNA viruksia). DNA-kaksoisjuoste muodostuu kahdesta yhteen liitetystä neljän eri emäksen, adeniinin (A), sytosiinin (C), tyymiinin (T) ja guaniinin (G), ketjusta. Nämä kaksi ketjua ovat suurimmaksi osaksi toistensa peilikuvia ja liittyvät yhteen vetysidoksin siten, että A pariutuu aina T:n kanssa ja C G:n kanssa muistuttaen pitkiä tikapuita. Tämä DNA-kaksoisjuoste kiertyy ensin pituusakselinsa ympäri ja sitten histoni-alkuaineen ympärille, jotka taas pakkautuvat tiiviisti yhteen solun jakautumisen lähestyessä muodostaen kromosomin toisen puolen, jos kyseessä on diploidi eliö. Yksi kromosomi muodostuu siis kahdesta tietyn pituisesta pakkautuneesta DNA-kaksoisjuosteesta, jotka ovat liittyneet yhteen tietystä kohtaa solun jakautumisen ajaksi. Kromosomi muistuttaa muodoltaan pyykkipoikaa. Jokaisessa yksilön solussa on samat ja yhtä paljon kromosomeja. Kaikki yhden

solun kromosomit muodostavat yksilön genomien.

Jokainen DNA-kaksoisjuostemolekyyli sisältää suuren määrän perintötekijöitä eli geenejä. Geeni on pätkä DNA'a, joka koodaa erilaisia valkuaisaineita. Geeni ei koostu välttämättä vain valkuaisaineita koodaavasta emäsjärjestyksestä vaan myös emäsparijakoista, jotka leikataan pois geenistä muodostetusta kopiosta ennen valkuaisaineen koodausta.

DNA:n merkkialueet ja niiden käyttö tutkimuksissa

Koska jokaisen yksilön genomien sekvensointi ei ole vielä mahdollista, ja koska emme vielä tiedä jokaisen geenin rakennetta, niiden vuorovaikutuksia sekä tehtävää, perinnöllisissä tutkimuksissa käytetään usein hyväksi DNA merkkialueita. Merkkialueet ovat pieniä DNA pätkiä, ja ovat kuin kilometripylväitä DNA-ketjussa. Merkkialueet saattavat olla geenien sisällä tai niiden välissä. Merkkialueita käytetään hyväksi esimerkiksi yksilöiden perinnöllisen muuntelevuuden havainnoimiseksi sekä tutkimalla mitkä merkkialueista liittyvät tiettyihin fenotyyppisiin ominaisuuksiin.

Merkkialueet voidaan laittaa järjestykseen tekemällä risteytys kahden eri yksilön kesken ja analysoimalla tämän risteytyksen jälkeläistön merkkialueet, jotka eroavat vanhemmissa. Järjestys saadaan aikaiseksi arvioimalla, mitkä

merkkialueista käyttäytyvät samalla tavalla ja siten kytkeytyvät toisiinsa. Näin voidaan muodostaa koko kromosomin kattava merkkialueisiin perustuva kytkentäkartta.

Kytchentäkarttaa voidaan taasen käyttää hyväksi eri fenotyypisten ominaisuuksien kartoittamiseksi tiettyihin kromosomien osiin. Useat kaupallisesti tärkeät fenotyypiset ominaisuudet, kuten viljojen siementuotto tai lehmän maidontuotanto, ovat jatkuvia eli kvantitatiivisia ominaisuuksia. Näitä ei yleensä ohjaa vain yksi geeni, vaan niiden perustana on monta eri geeniä. Näitä kvantitatiivisia ominaisuuksia voidaan kartoittaa liittämällä niissä havaittu muuntelu merkkialueisiin (ns. kvantitatiivinen ominaisuuslokus eli QTL analyysi, jossa käytetään hyväksi risteytyksiä tai sitten yksinkertainen assosiaatioanalyysi, jossa suurta määrää eri linjoja tai populaatioita käytetään hyväksi). Tällätavoin perimän merkkialueet voivat mm. ennustaa sen, millaisia tuotteita tai miten paljon eri yksilöt tuottavat tulevaisuudessa. Kun merkkialueet on saatu liitettyä tiettyihin ominaisuuksiin (ns. QTL alueet), voidaan niitä käyttää myös hyväksi jalostuksessa. Tavanomaisessa jalostuksessa niitä voidaan käyttää eri risteytysmenetelmien kanssa varmistamassa, että haluttu alue kromosomia on mukana. Niitä voidaan myös käyttää itse ominaisuuksiin vaikuttavien geenien etsimiseen. Kun

haluttu geeni on löydetty, se voidaan siirtää haluttuun yksilöön parantamaan sen tuottoa.

Villiohran kytkentäkartta

Villiohran molekyyläarinen merkkialue kytkentäkartta tehtiin käyttäen risteytyksen 'Ashkelon x Mehola' F₂ populaatiota. Käytetyn Ashkelon-linjan alkuperäinen keräyspaikka on tienvarsi Välimeren rannalla sijaitsevan Ashkelon kaupungin laitamalla, kun taas Mehola kerättiin Jordan joen rannalta Kinneret järven ja Kuolleen Meren välimaastosta. Nämä kaksi lijaa valittiin niiden erilaisten ympäristöolosuhteiden ja kasvuominaisuuksien pohjalta. Kolmetoista mikrosatelliittia ja 22 AFLP alukeparia käytettiin 384 muuntelevan merkkialueen saamiseksi, joista loppujenlopuksi 202 pystyttiin kartoittamaan 11 kytkentäryhmään. Kahta kytkentäryhmää ei pystytty tunnistamaan minkään kromosomin osaksi, muut muodostivat osia kaikista ohran seitsemästä kromosomista. Kartoitettu alue oli 445 senttiMorgania (cM). Keskimääräinen etäisyys kahden merkkialueen välillä oli 2.2 cM. Jotta kartan kytkentäryhmät voitiin tunnistaa tunnettujen ohran kromosomien osiksi, 33 AFLP- ja 13 mikrosatelliittimerkkialuetta, jotka olivat aiemmin kartoitettu viljellyn ohran kytkentäkarttoihin, otettiin mukaan. Koska toinen vanhemmista oli yllättäen heterotsygoottinen monen merkkialueen suhteen, kytkentäkartan kattavuus on

arvioitu olevan suurimmillaan 55% ohran genomista. Tämä kytkentäkartta on ensimmäinen villiohran lajinsisäinen kartta.

Villiohran perinnöllinen, fenotyyppinen ja ympäristön muuntelu

Perinnöllinen ja fenotyyppinen muuntelu arvioitiin 72 linjan ja 21 villiohran luonnonpopulaation sisällä ja välillä. Nämä populaatiot ryhmitettiin lisäksi neljään erilaiseen ympäristötyyppiin eli 'ekotyyppiin'. Perinnöllisen ja fenotyyppisen muuntelun lisäksi ympäristömuuntelu arvioitiin eri ympäristömuuttujista, jotka kuvaavat paikallisia populaatioiden ympäristöolosuhteita. Perinnölliset, fenotyyppiset ja ympäristölliset etäisyydet arvioitiin eri linjojen ja populaatioiden välillä ja UPGMA dendrogrammit ('sukupuut') rakennettiin niiden pohjalta. Tulokset osoittivat, että perinnöllinen muuntelu oli suurempaa populaatioiden välillä kuin niiden sisällä, ja siten populaatorakenne havaittiin odotetusti. Fenotyyppinen muuntelu oli taasen täysin päinvastaisesti enemmän populaatioiden sisällä kuin välillä, eikä populaatorakennetta havaittu. Toisinsanoen eri populaatioiden välillä ei ollut niitä selkeästi eroittavia eroja. Perinnöllisten ja fenotyyppisten etäisyyksien väillä ei siten ollut mitään korrelaatiota. Korrelaatiota ei havaittu fenotyyppisen ja ympäristön

etäisyyksien välilläkään. Hyvin hienoinen korrelaatio havaittiin perinnöllisen ja ympäristön etäisyyksien välillä. Kaksikymmentäkolme AFLP merkkialuetta tunnistettiin ekotyyppispesifiseksi, eli niiden muuntelu oli erilainen eri ekotyyppien välillä. Näistä viisi oli kartoitettu. Neljä näistä kartoitetuista ekotyyppispesifisestä merkkialueesta korreloi sekä ympäristön että fenotyyppisten ominaisuuksien kanssa. Näin havaittiin suora yhteys perimän, fenotyypin ja ympäristön kanssa. Tarkemmat mikroklimaattiset ympäristötiedot ja epäoptimaalisissa olosuhteissa mitatut fenotyyppiset ominaisuudet mahdollistaisivat paremman ympäristön, perimän ja fenotyyppisten ominaisuuksien vertailun.

Villiohran assosiaatioanalyysi ja sen vertailu aiemmin löydettyihin QTL alueisiin

Aiemmassa tutkimuksessa havaittuja QTL alueita vertailtiin merkkialueiden ja fenotyyppisten ominaisuuksien assosiaatioihin, jotka oli arvioitu 81 villiohralinjassa. Näissä linjoissa mitattiin 33 eri fenotyyppistä kasvuun, hiili ja typpiekonomiaan sekä morfologiaan liittyvää ominaisuutta. Linjat genotypattiin ja tässä tutkimuksessa käytettiin 68 AFLP merkkialuetta, jotka oli aiemmin kartoitettu viljellyn ohran ja villiohran kytkentäkartoille. Näistä 68

merkkialueesta 60 korreloi tilastollisesti merkittävästi yhden tai useamman fenotyypin ominaisuuden kanssa. Useat näistä merkkialue-ominaisuus assosiaatioista tukivat aiemmin havaittuja QTL alueita. Merkkialue-suhteellinen kasvunopeus -assosiaatiot tukivat kahta neljästä suhteellisen kasvunopeuden QTL alueesta. Neljä merkkialuetta, jotka liittyvät kasvin yhteyttämisenopeuteen, kartoitettiin kromosomiin 4, johon oli kartoitettu myös yhteyttämisenopeuden ja lehden ilmarakojen konduktanssin QTL alueet. Yksi näistä neljästä merkkialueista korreloi myös viherhiukkasmäärän kanssa, ehdottaen, että tämä kromosomialue saattaa olla hyvinkin tärkeä kasvin yhteyttämiseen liittyville ominaisuuksille. Yksi spesifisen lehtialan QTL alue kartoitettiin kromosomiin 7. Tätä QTL aluetta tuki merkkialue-spesifinen lehtiala assosiaatio. Spesifinen lehtiala on tärkeä suhteellisen kasvunopeuden osakomponentti. Merkkialueet kromosomeissa 1, 2 ja 4 liittyivät vahvasti lehden paksuuteen ja leveyteen sekä siemenpainoon, näin ehdottaen, että nämä ominaisuudet ovat perinnöllisesti kytkeytyneitä toisiinsa.

Villiohnan siemenen itämislepoon liittyvät QTL alueet

QTL analyysi suoritettiin villiohnan siemenen itämislevon perinnöllisen

taustan tutkimiseksi. Kun siemen on itämislepovossa, se ei idä, vaikka se olisi tarpeeksi kypsä ja vaikka olosuhteet itämiselle olisivat optimaaliset. Useimmat viljellyt ohralajikkeet ovat menettäneet itämisleponsa lähes kokonaan. Tietynasteinen itämislepo on kuitenkin tärkeä, koska ilman sitä siemen saattaa itää tähkässä ennen sadonkorjuuta. Luonnossa itämislepo on erittäin tärkeä varsinkin epävakaaassa ympäristössä, jolloin itäminen väärään aikaan saattaa aiheuttaa taimen kuoleamisen. Liian voimakas itämislepo viljellyssä ohrassa on taasen myös huono asia, koska siemenet täytyy säilöä tietynlaisessa ympäristössä kunnes itämislepo saadaan poistettua. Liian voimakas itämislepo aiheuttaa myös rikkaruohoeffektin pelloilla, joilla seuraava viljelty laji ei ole samanlajikkeinen ohra. Tämän tutkimuksen villiohnan siementen itävyys testattiin F_4 populaatioissa. Yhteensä viisi QTL aluetta havaittiin eri itävyytsteisteissä. Nämä QTL alueet sijoittuivat kromosomeihin 1, 2, 5, 6 ja 7. Eri QTL alueet selittivät 8% - 25% itävyyden kokonaisuuntelusta. Ashkelon alleelit nostivat itävyyttä paitsi kromosomi 5'n QTL alue, jossa Mehola alleeli paransi itävyyttä.

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Acknowledgements

In many ways plants are easier to work with than animals. For example they can't run away or make noises when you are taking a sample... But there is the other side that I found out when I was spending endless hours in the greenhouses bagging plants, planting, tying them to canes or taking samples. In hot summer weather the greenhouse was pretty much a good substitute for sauna. Although, alas, there was no lake within a jumping distance.

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A year or two before my contract was to end, we started thinking where to go afterwards. I liked living in the Netherlands, but missed the space and nature dearly. So, we decided upon Edinburgh. My husband found a job in Roslin and I found my way to Deborah Charlesworth with the help of Peter Visscher. Thank you Deborah, for taking me in with the burden of my 'unfinished business'. I have thoroughly enjoyed (and still am) working in Edinburgh among lovely colleagues, thanks for bearing with me during the last part of this thesis!

As many people, who come into contact with me, know, work is not everything to me. A big chunk of my spare time is spend with horses (and if not horses, it'll be growing things in the garden, and if not garden, it'll be walking the hills...). Luckily I managed to lure my husband into the hobby as well. Riding and just taking care of the horses has been the perfect stress-releaser. With horses you will have to give your undivided attention to them and leave the working day behind. The social circles in Whiteloch Farms riding

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*The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say.*

*The Road goes ever on and on
Out from the door where it began.
Now far ahead the Road has gone,
Let others follow it who can!
Let them a journey new begin,
But I at last with weary feet
Will turn towards the lighted inn,
My evening-rest and sleep to meet.*

*Still round the corner there may wait
A new road or a secret gate;
And though I oft have passed them by,
A day will come at last when I
Shall take the hidden paths that run
West of the Moon, East of the Sun.*

JRR Tolkien



Curriculum vitae



Tytti Kaarina Vanhala was born in June the 18th 1971 in Saarijärvi, Finland, in the middle of thousands of lakes and hectares of forests. She went to the comprehensive school in Saarijärvi 1978 – 1987. After the basic education she continued to study in Lukio in Saarijärvi and graduated in 1990. Until the last exams she was unsure where to continue her studies, favourites were history (archaeology) and information science (the one to do with libraries, not computers). But then, at the last moment, she realised that what really interested her was biology. She took her entrance exams in mind to study cell and molecular biology in the University of Jyväskylä, but got into the University of Oulu instead. In the meanwhile she was also admitted to a professional school to study to be a seamstress. But in the end life in science lured her more than career in fashion. In Oulu she chose to study genetics, and was not deterred even after the first genetics courses involving sucking *Drosophilas* out of their bottles with glass tubes... Wanting to work in animal genetics she did her practical period in the Agricultural Research Centre of Finland in Jokioinen during the summer of 1993. Her main research there consisted of typing bulls for their kappa-kasein locus. In the autumn of 1994 she returned to Jokioinen to start her Master's thesis research. Her task was to look into the genetic variation within and between several chicken lines using microsatellite markers. Towards the end of the year, she decided it was time for some foreign experience before graduation and she applied to go to the Trinity College in Dublin for an Erasmus exchange. Unfortunately their quota was filled, and in the autumn of 1995 she went to Wageningen University, The Netherlands, instead. During her 9 months stay at the department of Animal Breeding and Genetics, she helped to find and develop more microsatellite markers in the chicken genome. After the stay in the Netherlands she finished her studies in the University of Oulu, formally graduating on 7th of April 1997. Before that she had already moved back to Wageningen following her boyfriend's work. In October 1997 she started as an OIO in the department of Plant Breeding in Wageningen, the results of the research being accounted in this thesis. In the end of September 2001 she moved to Cowbraehill, Scotland, on a side of a hill amongst the sheep. She started working with sequence diversity in *Antirrhinum*, and later *Silene*, in the Institute for Cell, Animal and Population Biology, University of Edinburgh, Scotland. At first she was funded by the Marie Curie fellowship for half a year, and subsequently by Leverhulme Trust.

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